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(54) Title: CONTINUOUS POROUS MATRIX ARRAYS

(57) Abstract

Arrays of polymers on a continuous porous matrix array and methods of making and using same are disclosed.

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CONTINUOUS POROUS MATRIX ARRAYS

Cross Reference to Related Applications

This application claims the benefit of priority of Provisional Application U.S.S.N. 60/125,954, filed March 24, 1999.

Background of the Invention

Rapid advances in the ability to accurately determine polynucleotide sequences, such as DNAs and RNAs from the genomes of organisms, has made possible the sequencing of huge quantities of polynucleotides. In recent years, the entire genomes of microorganisms, such as Helicobacter pylori, have been sequenced.

Traditional sequencing methods have relied on automated sequencing equipment which processes a polynucleotide strand one base at a time. A more recent approach, sequencing by hybridization (SBH), which could potentially increase sequencing throughput, relies on fragmenting a target polynucleotide into short segments; these short segments can be captured, for example on an ordered array of immobilized complementary strands, and the sequences of the individual fragments determined. Alignment of the fragment sequences, typically with the aid of a computer for longer sequences of interest, provides the sequence of the target polynucleotide. See, for example, U.S. Patent No. 5,552,270 to Khrapko et al.

Arrays of immobilized polynucleotides have been constructed for use in SBH techniques. However, new types of arrays, and methods for making them, are needed.

Summary of the Invention

In general, the invention features, an array having a plurality of addresses, each address of the plurality being positionally distinguishable from each other address of the

plurality; and each of said plurality of addresses being located on, or in, a continuous porous matrix, e.g., a polymer matrix or gel, e.g., a polyacylamide gel, agarose gel, dextran gel or polysaccharide gel. As the continuous porous matrix is three dimensional, reagents can be deposited on the surface of the matrix, within the matrix, or both.

The unique reagents can be deposited, as a liquid, on the continuous porous matrix array. A dispensing device, e.g., a piezo-dispenser, which can deposit small volumes, e.g., volumes of less than 1,000 picoliters can be used to deliver the reagent solution. The delivery of small volumes allows for relatively high density of arrays, preferably arrays with densities equal to or greater than 50, 100, or 200 addresses/cm^2.

In a preferred embodiment, the continuous porous matrix includes about 1-20%, more preferably 2-10%, more preferably about 5% polymer, e.g,. acrylamide, dextran, agarose or saccharide.

In a preferred example, a unique reagent is delivered in a liquid to the continuous porous matrix and is imbibed into the matrix.

In a preferred embodiment, the area or volume of the continuous porous matrix occupied by a unique reagent at an address should be continuous, as opposed to being separate unconnected chambers or pores. In another preferred embodiment, the area or volume of the continuous porous matrix occupied by a plurality of addresses is continuous, as opposed to being separate unconnected chambers or pores.

In preferred embodiments, the piezo-delivered reagent imbibes into the matrix and occupies a three dimensional space (as opposed to merely occupying the surface.

In one embodiment, each of said plurality of addresses includes a unique reagent, e.g., a unique capture probe.

In another embodiment, the unique reagents are covalently coupled to the continuous porous matrix.

In still another embodiment, the unique reagents are not organized by one or more of size, charge, or mobility.

In another embodiment, there is an area of the matrix, between the unique reagents of the plurality, where the matrix is substantially free of unique reagents.

In a preferred embodiment, the plurality of addresses includes at least 10, 100, 500, 1,000, 5,000, or 10,000 addresses.

In a preferred embodiment, the plurality of addresses includes less than 10, 100, 500, 1,000, 5,000, or 10,000 addresses.

In a preferred embodiment, the plurality of addresses includes less than 9, 99, 499, 999, 4,999, or 9,999 addresses.

In a preferred embodiment, the array density, i.e., the number of addresses per square centimeter on the continuous porous matrix, or in a plurality of addresses on the continuous porous matrix, is at least 10, more preferably 50, more preferably 100, more preferably 200, more preferably 500, more preferably 1,000, more preferably 2000, more preferably 10,000, or even more preferably 2000 addresses per square centimeter of continuous porous matrix surface.

In a particularly preferred embodiment, the density is greater than 5, more preferably 10, more preferably 50, more preferably 100 or more preferably 200 addresses/cm^2, but less than 2,000, 10,000, or 20,000 addresses/cm^2.

In a preferred embodiment, the array density, i.e., the number of addresses on the continuous porous matrix, or in a plurality of addresses on the continuous porous matrix, is less than 10, more preferably 100, more preferably 500, more preferably 1,000, or even more preferably 10,000, addresses per square centimeter of continuous porous matrix surface.

In a preferred embodiment, the array density, i.e., the number of addresses on the continuous porous matrix, or in a plurality of addresses on the continuous porous matrix,

is less than 9, more preferably 99, more preferably 499, more preferably 999, or even more preferably 9,999, addresses per square centimeter of continuous porous matrix surface.

In a preferred embodiment, the continuous porous matrix has one or more of the following characteristics: it includes a polyacrylamide gel; the gel is mounted on a rigid support, preferably glass or silanized glass; the gel includes groups reactive with primary amines, e.g., to allow reaction with derivatized DNA, RNA, or protein; the gel is 1-500 m, preferably 5-250 m, more preferably 5-100 m, even more preferably 10-100 m, even more preferably 10-50 m, and most preferably about 20 m thick; the area on or in which the address are located is 10-1,000 mm^2, preferably 50-500 mm^2, more preferably 50-250 mm^2, even more preferably 125 mm^2.

A substance, e.g., a unique reagent, e.g., a capture probe, can be deposited on the array, e.g., with a device which expels the unique reagents, e.g., with a piezo-dispensing device.

In a preferred embodiment, an address includes a capture probe, such as a nucleic acid, e.g., an oligonucleotide, a polysaccharide, a protein, e.g., an enzyme or an antibody, or a substrate or ligand of a protein. The capture probe will preferably have a unique (i.e., not repeated in another nucleic acid of the plurality) region.

In a preferred embodiment, the porous matrix has a continuous surface.

In a preferred embodiment the porous array is disposed on a support, e.g., a porous or nonporous material. A suitable nonporous material is glass or silicon. The support can be flexible or non-flexible.

In a preferred embodiment, the plurality of addresses occupies a portion of a continuous porous array, which has a continuous surface. In a preferred embodiment, at least 2, and preferably 10, 50, 500, 1,000, 5,000 or 10,000 addresses are located in a region having a continuous surface.

In a preferred embodiment, the reagent is delivered to the matrix in a volume of less than 100, 200, 300, 400, 500, 1,000, or 5,000 picoliters.

In a preferred embodiment: a reagent will occupy about 0.0025-0.25 mm^2, more preferably about 0.005-0.125 mm^2, more preferably about 0.010-0.075 mm^2, more preferably about 0.020-0.050 mm^2, and more preferably about 0.25 mm^2 of an array; the center-to-center distance between two addresses of a plurality is about 25 microns to 2 millimeters, more preferably about 50 microns to 1 millimeter, more preferably about 100 microns to 500 microns, more preferably about 250-400 microns.

In a preferred embodiment, the diameter of the area covered by a unique reagent is between 30-1,000 microns, more preferably between 30-500 microns, more preferably between about 100-500 microns, more preferably about 100-250 microns, more preferably about 30 or 180 microns.

In a preferred embodiment, the unique reagent is delivered to the continuous porous matrix as a liquid, and the volume delivered to an address is less than 500 nanoliters (nl), more preferably less than 500 nl, more preferably less than 450 nl, more preferably less than 200 nl, or more preferably less than 100 nl. In a particularly preferred embodiment, the volume delivered is equal to, or less than, 1,000, 500, 400, 325, 250, 200, 150, 100 or 30 picoliters. Generally, smaller volumes are desirable as they allow for arrays of higher chemistry.

The reagent at an address can be delivered in a single aliquot, e.g., a discharge or droplet, or in multiple aliquots, e.g., multiple discharges or droplets.

In a preferred embodiment, a unique reagent is synthesized *in situ*, on or in the matrix, or, is delivered preformed to the matrix.

In another aspect, the invention features, a method of making an array having a plurality of unique reagents disposed therein or thereon. The method includes: (1) providing an array described herein (e.g., an array having a plurality of addresses, each

address of the plurality being positionally distinguishable from each other address of the plurality; and each of said plurality of addresses being located on or in a continuous porous matrix) and; (2) introducing a first unique reagent, e.g., a first capture probe at a first address; and (3) introducing a second unique reagent, e.g., a second capture probe at a second address, thereby providing an array having a plurality of unique reagents disposed therein or thereon.

In a preferred embodiment, the method includes: (1) providing a continuous porous matrix; (2) applying a first reagent comprising a capture probe on said porous matrix; (3) optionally, displacing a dispenser for dispensing of a reagent relative to a surface of the matrix (the same, or different dispenser can be used to dispense the first and second unique reagent); (4) applying a second reagent comprising a second or subsequent capture probe to the continuous porous matrix; and (5) repeating steps 3, or optionally 3 and 4, or until an array of capture probes having diverse sequences are formed.

In preferred embodiments, the continuous porous matrix includes a polymer matrix or gel, e.g., a polyacrylamide, polysaccharide, or agarose gel.

This method can be used to form an array of addresses, which addresses may be 2 or 3 dimensional, wherein each of the addresses is positionally distinguishable from other addresses on the array, and wherein each positionally distinguishable probe includes a unique (i.e., not repeated in another probe) region.

The unique reagents can be deposited, as a liquid, on the continuous porous matrix array. A dispensing device, e.g., a piezo-dispenser, which can deposit small volumes, e.g., volumes of less than 1,000 picoliters can be used to deliver the reagent solution. The delivery of small volumes allows for relatively high density of arrays, preferably arrays with densities equal to or greater than 50, 100, or 200 addresses/cm^2.

In a preferred embodiment, the continuous porous matrix includes about 1-20%, more preferably 2-10%, more preferably about 5% polymer, e.g,. acrylamide, dextran, agarose or saccharide.

In a preferred example, the unique reagent is delivered in a liquid to the continuous porous matrix and is imbibed into the matrix.

In a preferred embodiment, the area or volume of the continuous porous matrix occupied by a unique reagent at an address should be continuous, as opposed to being separate unconnected chambers or pores. In another preferred embodiment, the area or volume of the continuous porous matrix occupied by a plurality of addresses is continuous, as opposed to being separate unconnected chambers or pores.

In preferred embodiments, the piezo-delivered reagent imbibes into the matrix and occupies a three dimensional space (as opposed to merely occupying the surface.

In one embodiment, each of said plurality of addresses includes a unique reagent, e.g., a unique capture probe.

In another embodiment, there is an area of the matrix, between the unique reagents of the plurality, where the matrix is substantially free of unique reagents.

In a preferred embodiment, the plurality of addresses includes at least 10, 100, 500, 1,000, 5,000, or 10,000 addresses.

In a preferred embodiment, the plurality of addresses includes less than 10, 100, 500, 1,000, 5,000, or 10,000 addresses.

In a preferred embodiment, the plurality of addresses includes less than 9, 99, 499, 999, 4,999, or 9,999 addresses.

In another embodiment, the unique reagents are covalently coupled to the continuous porous matrix.

In still another embodiment, the unique reagents are not organized by one or more of size, charge, or mobility.

In a preferred embodiment, the array density, i.e., the number of addresses per square centimeter on the continuous porous matrix, or in a plurality of addresses on the continuous porous matrix, is at least 10, more preferably 50, more preferably 100, more preferably 200, more preferably 500, more preferably 1,000, more preferably 2000, more preferably 10,000, or even more preferably 2000 addresses per square centimeter of continuous porous matrix surface.

In a particularly preferred embodiment, the density is greater than 5, more preferably 10, more preferably 50, more preferably 100 or more preferably 200 addresses/cm^2, but less than 2,000, 10,000, or 20,000 addresses/cm^2.

In a preferred embodiment, the array density, i.e., the number of addresses on the continuous porous matrix, or in a plurality of addresses on the continuous porous matrix, is less than 10, more preferably 100, more preferably 500, more preferably 1,000, or even more preferably 10,000, addresses per square centimeter of continuous porous matrix surface.

In a preferred embodiment, the array density, i.e., the number of addresses on the continuous porous matrix, or in a plurality of addresses on the continuous porous matrix, is less than 9, more preferably 99, more preferably 499, more preferably 999, or even more preferably 9,999, addresses per square centimeter of continuous porous matrix surface.

In a preferred embodiment the continuous porous matrix has one or more of the following characteristics: it includes a polyacrylamide gel; the gel is mounted on a rigid support, preferably glass or silanized glass; the gel includes group reactive with primary amines, to allow reaction with derivatized DNA, RNA, or protein; the gel is 1-500 m, preferably 5-250 m, more preferably 5-100 m, even more preferably 10-100 m, even more preferably 10-50 m, and most preferably about 20 m thick; the area on or in

which the address are located is 10-1,000 mm^2, preferably 50-500 mm^2, more preferably 50-250 mm^2, even more preferably 125 mm^2.

A substance, e.g., a unique reagent, e.g., a capture probe, can be deposited on the array, e.g., with a device which expels the unique reagents, e.g., with a piezo-dispensing device.

In a preferred embodiment the porous array is disposed on a support, e.g., a porous or nonporous material. A suitable nonporous material is glass or silicon. The support can be flexible or non-flexible.

In a preferred embodiment, the plurality of addresses occupies a portion of a continuous porous array, which has a continuous surface. In a preferred embodiment, at least 2, and preferably 10, 50, 500, 1,000 or 10,000 addresses are located in a region having a continuous surface.

In a preferred embodiment, the reagent is delivered to the matrix in a volume of less than 100, 200, 300, 400, 500, 1,000, or 5,000 picoliters.

In a preferred embodiment: a reagent will occupy about 0.0025-0.25 mm^2, more preferably about 0.005-0.125 mm^2, more preferably about 0.010-0.075 mm^2, more preferably about 0.020-0.050 mm^2, and more preferably about 0.25 mm^2 of an array; the center-to-center distance between two addresses of a plurality is about 25 microns to 2 millimeters, more preferably about 50 microns to 1 millimeter, more preferably about 100 microns to 500 microns, more preferably about 250-400 microns.

In a preferred embodiment, the diameter of the area covered by a unique reagent is between 30-1,000 microns, more preferably between 30-500 microns, more preferably between about 100-500 microns, more preferably about 100-250 microns, more preferably about 30 or 180 microns.

In a preferred embodiment, the unique reagent is delivered to the continuous porous matrix as a liquid, and the volume delivered to an address is less than 500

nanoliters (nl), more preferably less than 500 nl, more preferably less than 450 nl, more preferably less than 200 nl, or more preferably less than 100 nl. In a particularly preferred embodiment, the volume delivered is equal to, or less than, 1,000, 500, 400, 325, 250, 200, 150, 100 or 30 picoliters. Generally, smaller volumes are desirable as they allow for arrays of higher chemistry.

The reagent at an address can be delivered in a single aliquot, e.g., a discharge or droplet, or in multiple aliquots, e.g., multiple discharges or droplets.

In a preferred embodiment, a unique reagent is synthesized *in situ*, on or in the matrix, or, is delivered preformed to the matrix.

In a preferred embodiment, a unique reagent is synthesized *in situ*, on or in the matrix, or, is delivered preformed to the matrix.

In another aspect, the invention features, a method of analyzing a sample. The method includes: (1) providing an array described herein (e.g., an array having a plurality of addresses, each address of the plurality being positionally distinguishable from each other address of the plurality; and each of said plurality of addresses being located on or in a continuous porous matrix, each address of the plurality having a unique reagent, e.g., a capture probe); (2) contacting the sample, or a molecule in the sample, with said array; (3) evaluating an interaction of the sample with a reagent at least one address of the plurality thereby analyzing the sample.

In preferred embodiments, the sample includes a polynucleotide sequence which is: a DNA molecule: all or part of a known gene; wild type DNA; mutant DNA; a genomic fragment, particularly a human genomic fragment; a cDNA, or a human cDNA.

In a preferred embodiment: an interaction of a sample, preferably a molecule, or molecules in the sample, with at least 1, 10, 100, 5,000, or 1,000, additional addresses is evaluated; a signal from one address is compared to a signal from a second address; at

least one address provides a standard or control signal; a data corresponding to a signal from at least one and preferably at least 10, 50, 100, 500, 1,000, 5,000 or 10,000 addresses is stored in a computer-readable form.

In a preferred embodiment, an address is evaluated by detecting or measuring a signal, e.g., a spectrophotometer signal, e.g., a fluorescent signal. In a preferred embodiment, the reagent is a nucleic acid and a sample containing nucleic acids is contacted with the array. In a particularly preferred embodiment, hybridization of a sample, a molecule, e.g., a nucleic acid, to a reagent, e.g., a nucleic acid capture probe, at an address, allows a signal, e.g., a spectrophotometer signal, e.g., a fluorescent signal, to be generated by that address.

In a preferred embodiment the method includes diagnosing a disorder, staging a disorder, evaluating the efficacy of a treatment, or generally, analyzing a sample. The disorder can be a proliferative disorder, e.g., a malignant disorder, a genetically conditioned disorder in a subject. In such embodiments methods include: providing a sample, e.g., a disease or disorder specific sample, e.g., a cancerous sample, e.g., tissue, a bodily fluid, e.g., urine, blood or CSF, a biopsy, from said subject; evaluating the expression of one or more selected genes by contacting said sample with, an array of capture probes described herein, e.g., an array of nucleic acid or polypeptide capture probes, wherein the level of expression, e.g., a change, e.g., an increase or decrease in the level of expression, e.g., in the level of the selected genes or gene products, relative to a control, diagnosis the disorder, indicates a stage in the disorder, or indicates the efficacy of a treatment.

In preferred embodiments the method is performed: on a sample from a mammal, a sample from a human subject; a sample from a cancer patient; to determine if the individual from which the sample is taken should receive a drug or other treatment; to diagnose an individual for a disorder or for predisposition to resistance to treatment, to

stage a disease or disorder.

In a particularly preferred embodiment the method further includes choosing a therapeutic modality, e.g., a particular anticancer treatment, or a dosage thereof, based on the level of expression of a selected gene.

In another aspect, the invention features, a method for evaluating the effectiveness of a treatment, e.g., the administration of a compound, for treating a disorder, e.g., a proliferative disorder, e.g., a malignant disorder, genetically conditioned disorder, or a disorder such as diabetes, Aids or cancer . The method includes: providing a cell, e.g., a cultured cell, a transformed cell, a cell from a cancer, or a test organism; administering said treatment to said cell (or test organism); and evaluating the expression of one or more selected genes, e.g., by contacting a sample from said cell (or test organism) with an array of capture probes described herein, e.g., an array of nucleic acid or polypeptide probes; wherein a change, e.g., decrease, in the level of said one or more selected genes or gene products in a sample given said treatment, e.g., relative to the level of expression without the treatment, indicative of the effectiveness of the compound for treating said disorder.

In preferred embodiments the sample includes a polynucleotide sequence which is an RNA molecule, e.g., an RNA transcript; a wild type RNA; a mutant RNA; or a human RNA.

In preferred embodiments the sample includes a polynucleotide sequence which is: a human sequence; a non-human sequence, e.g., a mouse, rat, pig, primate.

In a preferred embodiment the sample is a nucleic acid, e.g., mRNA, cDNA or genomic DNA from a selected sample, e.g., a tissue or cell.

In preferred embodiments the capture probes are single stranded probes in an array.

In preferred embodiments the method is performed: on a sample from a human subject; and a sample from a prenatal subject; as part of genetic counseling; to determine if the individual from which the target nucleic acid is taken should receive a drug or other treatment; to diagnose an individual for a disorder or for predisposition to a disorder; to stage a disease or disorder.

In a preferred embodiment, the interaction of the sample with a capture probe is hybridization or binding, and that interaction is evaluated by evaluating a signal, the production of which is dependent on the interaction, e.g., hybridization or binding of a labeled sample molecule to a capture probe.

In preferred embodiments the unique reagents have a structure comprising a double stranded portion and a single stranded portion in an array.

In preferred embodiments hybridization to the array is detected by mass spectrophotometry, e.g., by MALDI-TOF mass spectrophotometry.

In a preferred embodiment, the method includes one or more steps which allow evaluation of an interaction between the sample and a unique reagent. Thus, in a preferred embodiment, amplified sample sequence, which hybridizes to a capture probe, or sample, is the substrate of or template for an enzyme mediated reaction. For example, after hybridization to the capture probe, the amplified sample sequence is ligated to the capture probe, or after hybridization it is extended along the capture probe.

In preferred embodiments, the method includes one or more enzyme mediated reactions in which a nucleic acid used in the method, e.g., an amplified sample sequence, a capture probe, a sequence to be analyzed, or a molecule which hybridizes thereto, is the substrate or template for the enzyme mediated reaction. The enzyme mediated reaction can be: an extension reaction, e.g., a reaction catalyzed by a polymerase; a linking reaction, e.g., a ligation, e.g., a reaction catalyzed by a ligase; or a nucleic acid cleavage reaction, e.g., a cleavage catalyzed by a restriction enzyme, e.g., a Type IIs enzyme. The

amplified sample sequence which hybridizes with the capture probe can be the substrate in an enzyme mediated reaction, e.g., it can be ligated to a strand of the capture probe or it can be extended along a strand of the capture probe. Alternatively, the capture probe can be extended along the hybridized amplified sample sequence. (Any of the extension reactors discussed herein can be performed with labeled, or chain terminating, subunits.) The capture probe duplex can be the substrate for a cleavage reaction. These reactions can be used to increase specificity of the method or to otherwise aid in detection, e.g., by providing a signal.

In preferred embodiments, the method includes providing a continuous porous array having a plurality of capture probes, wherein each of the capture probes is (1) positionally distinguishable from the other capture probes of the plurality and has a unique variable region (not repeated in another capture probe of the plurality), (2) has a variable region capable of hybridizing adjacent to the genetic event; and has a 3' end capable of serving as a priming site for extension; hybridizing the amplified sample sequence having a genetic event to a capture probe of the array, (preferably the region of the amplified sample sequence having a genetic event hybridizes adjacent to the variable region of a capture probe); and using the 3' end of the capture probe to extend across the region of genomic nucleic acid having a genetic event with one or more terminating base species, where if more than one is used each species has a unique distinguishable label e.g. label 1 for base A, label 2 for base T, label 3 for base G, and label 4 for base C; thereby analyzing the amplified sample sequence.

In a preferred embodiment, at least one reaction step is performed in the continuous porous matrix, as described herein.

The unique reagents can be deposited, as a liquid, on the continuous porous matrix array. A dispensing device, e.g., a piezo-dispenser, which can deposit small volumes, e.g., volumes of less than 1,000 picoliters can be used to deliver the reagent

solution. The delivery of small volumes allows for relatively high density of arrays, preferably arrays with densities equal to or greater than 50, 100, or 200 addresses/cm^2.

In a preferred embodiment, the continuous porous matrix includes about 1-20%, more preferably 2-10%, more preferably about 5% polymer, e.g,. acrylamide, dextran, agarose or saccharide.

In a preferred example, the unique reagent is delivered in a liquid to the continuous porous matrix and is imbibed into the matrix.

In a preferred embodiment, the area or volume of the continuous porous matrix occupied by a unique reagent at an address should be continuous, as opposed to being separate unconnected chambers or pores. In another preferred embodiment, the area or volume of the continuous porous matrix occupied by a plurality of addresses is continuous, as opposed to being separate unconnected chambers or pores.

In preferred embodiments, the piezo-delivered reagent imbibes into the matrix and occupies a three dimensional space (as opposed to merely occupying the surface.

In one embodiment, each of said plurality of addresses includes a unique reagent, e.g., a unique capture probe.

In another embodiment, the unique reagents are covalently coupled to the continuous porous matrix.

In still another embodiment, the unique reagents are not organized by one or more of size, charge, or mobility.

In another embodiment, there is an area of the matrix, between the unique reagents of the plurality, where the matrix is substantially free of unique reagents.

In a preferred embodiment, the plurality of addresses includes at least 10, 100, 500, 1,000, 5,000, or 10,000 addresses.

In a preferred embodiment, the plurality of addresses includes less than 10, 100, 500, 1,000, 5,000, or 10,000 addresses.

In a preferred embodiment, the plurality of addresses includes less than 9, 99, 499, 999, 4,999, or 9,999 addresses.

In a preferred embodiment, the array density, i.e., the number of addresses per square centimeter on the continuous porous matrix, or in a plurality of addresses on the continuous porous matrix, is at least 10, more preferably 50, more preferably 100, more preferably 200, more preferably 500, more preferably 1,000, more preferably 2000, more preferably 10,000, or even more preferably 2000 addresses per square centimeter of continuous porous matrix surface.

In a particularly preferred embodiment, the density is greater than 5, more preferably 10, more preferably 50, more preferably 100 or more preferably 200 addresses/cm^2, but less than 2,000, 10,000, or 20,000 addresses/cm^2.

In a preferred embodiment, the array density, i.e., the number of addresses on the continuous porous matrix, or in a plurality of addresses on the continuous porous matrix, is less than 10, more preferably 100, more preferably 500, more preferably 1,000, or even more preferably 10,000, addresses per square centimeter of continuous porous matrix surface.

In a preferred embodiment, the array density, i.e., the number of addresses on the continuous porous matrix, or in a plurality addresses on the continuous porous matrix, is less than 9, more preferably 99, more preferably 499, more preferably 999, or even more preferably 9,999, addresses per square centimeter of continuous porous matrix surface.

In a preferred embodiment the porous array is disposed on a support, e.g., a porous or nonporous material. A suitable nonporous material is glass or silicon. The support can be flexible or non-flexible.

In a preferred embodiment, the plurality of addresses occupies a portion of a continuous porous array, which has a continuous surface. In a preferred embodiment,

at least 2, and preferably 10, 50, 100, 500, 1,000 or 10,000 addresses are located in a region having a continuous surface.

In a preferred embodiment, the reagent is delivered to the matrix in a volume of less than 100, 200, 300, 400, 500, 1,000, or 5,000 picoliters.

In a preferred embodiment: a reagent will occupy about 0.0025-0.25 mm^2, more preferably about 0.005-0.125 mm^2, more preferably about 0.010-0.075 mm^2, more preferably about 0.020-0.050 mm^2, and more preferably about 0.25 mm^2 of an array; the center-to-center distance between two addresses of a plurality is about 25 microns to 2 millimeters, more preferably about 50 microns to 1 millimeter, more preferably about 100 microns to 500 microns, more preferably about 250-400 microns.

In a preferred embodiment, the diameter of the area covered by a unique reagent is between 30-1,000 microns, more preferably between 30-500 microns, more preferably between about 100-500 microns, more preferably about 100-250 microns, more preferably about 30 or 180 microns.

In a preferred embodiment, the unique reagent is delivered to the continuous porous matrix as a liquid, and the volume delivered to an address is less than 500 nanoliters (nl), more preferably less than 500 nl, more preferably less than 450 nl, more preferably less than 200 nl, or more preferably less than 100 nl. In a particularly preferred embodiment, the volume delivered is equal to, or less than, 1,000, 500, 400, 325, 250, 200, 150, 100 or 30 picoliters. Generally, smaller volumes are desirable as they allow for arrays of higher chemistry.

The reagent at an address can be delivered in a single aliquot, e.g., a discharge or droplet, or in multiple aliquots, e.g., multiple discharges or droplets.

In a preferred embodiment, a unique reagent is synthesized *in situ*, on or in the matrix, or, is delivered preformed to the matrix.

In a preferred embodiment the continuous porous matrix has one or more of the following characteristics: it includes a polyacrylamide gel; the gel is mounted on a rigid support, preferably glass or silanized glass; the gel includes group reactive with primary amines, to allow reaction with derivatized DNA, RNA, or protein; the gel is 1-500 m, preferably 5-250 m, more preferably 5-100 m, even more preferably 10-100 m, even more preferably 10-50 m, and most preferably about 20 m thick; the area on or in which the address are located is 10-1,000 mm^2, preferably 50-500 mm^2, more preferably 50-250 mm^2, even more preferably 125 mm^2.

In a preferred embodiment, the method includes an amplification step, e.g: sample can be amplified prior to or upon contact with the array; interaction of a sample molecule with a capture probe, e.g., hybridization, can give rise to amplification of a molecule which is diagnostic of such interaction; or molecules eluted from the array can be amplified prior to further analysis, e.g., an analysis on a position array. Suitable amplification steps for nucleic acids include PCR, LCR, and rolling circle amplification.

In a preferred embodiment, the method includes amplifying a molecule which molecule is indicative of an interaction between the sample and a unique reagent, e.g., one or both of a capture probe or a sample nucleic acid at an address where the capture probe and sample nucleic acid have hybridized. Suitable amplification methods include PCR, LCR, and rolling circle amplification. In a preferred embodiment, the method can include: (1) providing a sample which includes a sample polynucleotide sequence (e.g., a test nucleotide), to be analyzed; (2)(a) contacting the sample with the array to allow annealing an effective amount of the sample polynucleotide sequence to capture probe and a single-stranded circular template, wherein the single-stranded circular template includes at least one copy of a nucleotide sequence complementary to the sequence of the sample polynucleotide sequence and optionally the presence of the sample probe circularizes the single stranded circular template, (b) combining the circular template

with an effective amount of at least two types of nucleotide triphosphates and an effective amount of a polymerase enzyme to yield a product, e.g., a single-stranded oligonucleotide multimer complementary to the circular oligonucleotide template; and optionally cleaving the product to produce cleaved amplified product, (3) analyzing said product or cleaved amplified product.

In a preferred embodiment, the rolling circle product is cleaved and the test nucleotide, or its complementary nucleotide, is sufficiently close to an end of the cleaved amplified product such that its presence can be detected by its effect on a reaction, which involves the end nucleotide of the cleavage product. In preferred embodiments, the sample nucleic acid serves as a primer. In other preferred embodiments synthesis is primed with a nucleic acid other than the sample nucleic acid.

In preferred embodiments, the test nucleotide, or its complementary nucleotide, is within 1 (i.e., it is at the end), 2, 3, 4 or 5 base pairs from the end of the cleaved amplified product.

Numerous variations in the circular oligonucleotide template can be used in embodiments which use rolling circle amplification. As described herein, the circular oligonucleotide template can be formed, e.g., in the reaction mix, from a linear precursor. As described herein, the circular oligonucleotide can be resistant to cleavage, providing for more efficient amplification.

In preferred embodiments, the circular oligonucleic acid template is prepared by a process which includes the steps of: (1) hybridizing each end of a linear precursor oligonucleotide to a positioning oligonucleotide, e.g., a capture probe sequence or a sample sequence, (wherein the positioning oligonucleotide has a 5' nucleotide sequence complementary to a portion of the sequence which includes the 3' end of the linear precursor oligonucleotide and a 3' nucleotide sequence complementary to a portion of the sequence which includes the 5' end of the linear precursor oligonucleotide, to yield an

open oligonucleotide circle wherein the 5' end and the 3' end of the open circle are positioned such that they can be joined, e.g., as to abut each other; and (2) joining the 5' end and the 3' end of the open oligonucleotide circle to yield a circular oligonucleotide template. Rolling circle amplification can be primed by the positioning oligonucleotide, e.g., the target nucleic acid, or by another in this or other methods disclosed herein.

In preferred embodiments, the oligonucleotide multimer formed by rolling circle amplification is more sensitive to cleavage than is the circular template. In preferred embodiments the oligonucleotide multimer is cleaved and the circular template is not cleaved. For example, in preferred embodiments the circular template has one or more nucleotide or modified nucleotide which is resistant to cleavage. While the modified nucleotide is resistant to cleavage, when it serves as a template, it nevertheless incorporation of the appropriate complementing nucleotide. The circular template, but preferably not the oligonucleotide multimer, can include one or more of deoxy uracil, or a methylated or hemimethylated base to render the circular template more resistant to cleavage.

Preferential cleavage of the oligonucleotide multimer can be effected by hybridization of a cleavage probe (thus forming a double stranded cleavage site) and said cleavage probe is chosen such that it cannot displace a strand from the circular template, thus allowing cleavage of only the oligonucleotide multimer.

Preferably, a circular template has about 15-1500 nucleotides, and more preferably about 24-500 nucleotides and most preferably about 30-150 nucleotides.

The oligonucleotide circular template itself may be constructed of DNA or RNA or analogs thereof. Preferably, the circular template is constructed of DNA. A liquid, e.g., a sample nucleic acid or protein binds to a portion of the circular template and is preferably single-stranded having about 4-50 nucleotides, and more preferably about 6-12 nucleotides.

In preferred embodiments the circular template includes a site for a type 2S restriction enzyme and the site is positioned, e.g., such that a type 2S restriction binding at the site cleaves adjacent the region which binds the sample sequence, cleaves in the region which binds the sample sequence, or cleaves at the target nucleotide.

In a preferred embodiment a region of the circular template is complementary to a genetic event, e.g., a mutation or SNP, and hybridizes differentially to a sample nucleic acid having the event and sample nucleic acid not having the event.

In preferred embodiments the reactions in one or more, and preferably all of steps 1, 2, and 3 are performed at the same temperature.

In preferred embodiments the reactions in one or more of steps 1, 2, and 3 are performed in the same container.

In preferred embodiments, analyzing a sample polynucleotide sequence includes, e.g., sequencing at least one nucleotide of the polynucleotide sequence, e.g., by sequencing by hybridization or positional sequencing by hybridization, detecting the presence of, or identifying, a genetic event, e.g., a SNP, in a target nucleic acid, e.g., a DNA.

In preferred embodiments, the genetic event is within 1, 2, 3, 4 or 5 base pairs from the end of the target molecule, or is sufficiently close to the end of the target molecule that a mismatch would inhibit DNA polymerase-based extension from a target/primed circle.

In preferred embodiments, the target nucleic acid is amplified, e.g., by PCR, prior to contact with a circular template.

The products of rolling circle amplification can be analyzed in various ways. In some embodiments, the products of rolling circle application are analyzed on positional arrays.

Accordingly, in preferred embodiments the method further includes: (3) analyzing a product from rolling circle amplification, e.g., by providing an array of a plurality of capture probes, wherein each of the capture probes is positionally distinguishable from other capture probes of the plurality on the array, and wherein each positional distinguishable capture probe includes a unique (i.e., not repeated in another capture probe) region; hybridizing the product with the array of capture probes, thereby analyzing the sample sequence.

In preferred embodiments, the array is a continuous porous matrix as described herein.

In preferred embodiments the method includes providing a plurality of single-stranded circular templates, wherein each of the single-stranded circular templates is positionally distinguishable from other single-stranded circular templates of the plurality on the array, and wherein each positional distinguishable single-stranded circular templates includes a unique (i.e., not repeated in another circular template) region complementary to sample target.

The polymerase enzyme can be any that effects the synthesis of the multimer, e.g., any polymerase described in U.S. Pat. No. 5,714,320. Generally, the definitions provided for circular vectors and their amplification in U.S. Patent No. 5,714,320, apply to terms used herein, unless there is a conflict between the terms in which case the meaning provided herein controls. U.S. Patent No. 5,714,320 and all other U.S. patents mentioned herein are incorporated by reference.

In preferred embodiments the primer, the sample, or the product is immobilized.

In preferred embodiments, the method further includes providing an effective amount of a cleavage primer which can hybridize to the oligonucleotide multimer, wherein the cleavage primer has at least one copy of a cleavage site flanked by a first and second detection moiety, and wherein the second detection moiety affects the

signal produced by the first moiety and upon cleavage at the cleavage site, the distance between the two moieties increases, resulting in an alteration of the signal, and cleaving the oligonucleotide multimer at the cleavage site to produce the cleaved amplified product;

and (3) analyzing said product from (2) b or c, e.g., thereby analyzing the sample sequence.

In preferred embodiments, the signal increases upon cleavage at the cleavage site.

In other preferred embodiments, the signal decreases upon cleavage at the cleavage site.

In preferred embodiments, the method further includes providing an effective amount of a first detection oligonucleotide having a first detecting moiety and a second oligonucleotide having a second detecting moiety, wherein the first and second oligonucleotides hybridize to the oligonucleotide multimer so that upon hybridization the first detection oligonucleotide hybridizes sufficiently close to the second detection oligonucleotide, such that the second detection moiety affects the signal produced by the first detection moiety.

A reagent can be dispensed on or in the continuous porous matrix using a liquid dispenser, a microvolume liquid handling system, or a piezo-dispensing device. The system should be capable of accurately verifying microvolume amounts of transfer liquid dispensed, e.g., by sensing a corresponding change in pressure in the microvolume liquid handling system, and should accurately measure an amount of dispensed liquid regardless of transfer liquid properties such as viscosity. An example of a dispensed reagent includes a capture probe as described herein. The system should also be able to sense pressure changes associated with clogging and misfiring to indicate such improper operation.

In a preferred embodiment, the microvolume liquid handling system prevents or minimizes clogging.

In one embodiment, the system includes four or fewer microdispensers are each coupled to a single positive displacement pump and pressure sensor.

In another embodiment, the system can verify that the transfer liquid is maintained within a given range of negative pressure (with respect to ambient atmospheric pressure) in order to accurately dispense microvolume amounts of transfer liquid and optimize the operation of the microdispenser.

In general, the microvolume liquid handling system should include a positive displacement pump operated by a stepper motor, a piezoresistive pressure sensor, and an electrically controlled microdispenser that utilizes a piezoelectric transducer bonded to a glass capillary. The microdispenser is capable of rapidly and accurately dispensing sub-nanoliter ("nl") sized droplets by forcibly ejecting the droplets from a small nozzle.

In another preferred embodiment of the microvolume liquid handling system, the number of microdispensers employed is equal to, or greater than, eight.

In another embodiment, the positive displacement pump (which includes a valve as described below), the stepper motor, and piezoresistive pressure sensor are replaced with a pressure control system for supplying system fluid and controlling system fluid pressure, a plurality of flow sensors for detecting fluid flow as well as pressure in the system fluid present in connecting tubing coupled to each microdispenser, and plurality of microfabricated valves, each microfabricated valve coupling each microdispenser to a system reservoir in the pressure control system.

In a preferred embodiment, the microdispenser can be mounted onto a 3-axis robotic system that is used to position the microdispenser at specific locations required to execute the desired liquid transfer protocol on or in a continuous porous matrix.

In a preferred embodiment, the system includes a system liquid and a transfer liquid in the dispensing system separated by a known volume of air ("air gap") which facilitates measuring small changes in pressure in the system liquid that correlate to the volume of transfer liquid dispensed. The transfer liquid contains the reagents being dispensed, e.g., one or more capture probes, while in one preferred embodiment the system liquid is deionized water. Each time a droplet in the microvolume dispensing range is dispensed, the transfer liquid will return to its prior position inside the microdispenser because of capillary forces, and the air gap's specific volume will be increased corresponding to the amount of transfer liquid dispensed. This has the effect of decreasing pressure in the system liquid line which is measured with a highly sensitive piezoresistive pressure sensor. The pressure sensor transmits an electric signal to control circuitry which converts the electric signal into a digital form and generates an indication of the corresponding volume of transfer liquid dispensed. An advantage of the present invention is its insensitivity to the viscosity of the transfer liquid. This is because the pressure change in the system liquid corresponds to the microvolume dispensed, without being dependent on the dispensed fluid viscosity. The present invention possesses unique capabilities in microvolume liquid handling. This system is capable of automatically sensing liquid surfaces, aspirating liquid containing reagents to be transferred, and then dispensing small quantities of liquid with high accuracy, speed and precision. The dispensing is accomplished without the dispenser contacting the continuous porous matrix. The system can positively verify the microvolume of liquid that has been dispensed during realtime operation.

In an aspect of the invention, the reagents, e.g., the capture probes are delivered to the continuous porous matrix by using a dispenser that prevents or minimizes clogging by activating the piezoelectric transducer at ultrasonic frequencies resonant with the microdispenser. By vibrating the microdispenser at its resonant ultrasonic frequency

during aspiration of transfer liquid into the glass capillary, clogging is prevented or minimized. The piezoelectric transducer is also activated at the same resonant ultrasonic frequencies when the capillary is being cleaned. The resonant vibrations of the capillary during cleaning result in a cleaner glass capillary interior than previously achieved. Because the same structure is used to prevent clogging, break up existing clogs, and clean the microdispenser, great efficiencies are achieved.

In a preferred embodiment, the microdispensers are positioned with a high degree of accuracy with regard to the surface of the continuous porous matrix. Visible or infrared light is transmitted through a porous matrix. When a particular microdispenser is moved from a first position to a second position, light passes through the glass capillary in the microdispenser where it is detected by a photodetector in optical contact with the glass capillary. The photodetector generates electronic signals corresponding to the amount of light received. The signals from the photodetectors are coupled to a computer which uses the signals to help locate and verify the position of the microdispenser.

A unique reagent, as referred to herein, is a reagent, which differs from a reagent at each other address in a plurality of addresses. The reagent can differ from the reagents at other addresses in terms of one or both of: structure, e.g., a sequence in the case of a nucleic acid or polypeptide; and function, e.g., the ability to interact with a substrate. Interaction can include binding, hybridization, or more generally an interaction, which results in the formation or breaking of a bond, e.g., a covalent or non-covalent chemical bond. A unique capture probe is one, which differs (at least) by structure, usually amino acid or nucleic acid sequence, from a capture probe at each other address of the plurality of addresses.

A unique reagent can be a molecule, e.g., a nucleic acid, a polypeptide, or a carbohydrate. The unique reagent can be or include a cell. Suitable cells include human or animal cells, genetically engineered cells, e.g., cells derived from transgenic

animals. Particularly desirable cells are those, which have been genetically engineered to express a reporter gene. Other useful cells include naturally occurring or genetically engineered cells, which secrete or express on the surface, a unique reagent.

A continuous matrix, as referred to herein, is a matrix, which uses a single matrix element, as opposed for example, devices which use a plurality of individual blocks of gel.

An address, as referred to herein, is a positionally distinct (from other addresses) portion of an array. By positionally distinct is meant that a reagent at a first address can be positionally distinguished form a reagent at a second address. The address is located in and/or on a continuous porous matrix. The address may be substantially two-dimensional, e.g., where a reagent is disposed essentially on the upper surface of the continuous porous array or three dimensional, e.g., where a reagent is disposed within the porous matrix.

A continuous porous matrix surface, as referred to herein, is a planar surface, which is free of surface features, e.g., indentations or raised structure, which separate individual addresses of a plurality of addresses. For example, it is free of ridges or troughs, e.g., ridges or troughs used to prevent movement of a reagent from one address to another.

The invention features arrays of a molecules, e.g., polymeric molecules such as nucleic acids and polypeptides, on a continuous porous matrix surface, e.g., a surface of a polymer matrix or a gel, e.g., a polyacrylamide, polysaccharide, or agarose gel, and methods for making and using the continuous porous matrix arrays. The arrays described herein can be used for sequencing by hybridization, e.g., in evaluating SNPs, or other SBH methods (e.g., where the arrays include nucleic acid strands immobilized to the gel), for cell based assays (e.g., where the arrays include, or are adjacent to and contacting, living cells), for expression profiling for genomics analysis for drug discovery or drug

target analysis, and for other uses which will be apparent to one of ordinary skill in the art.

In another aspect, the invention features, a continuous porous matrix, e.g., a continuous porous matrix described herein, which includes unique reagents which have been delivered to the continuous porous matrix, e.g., by a peizo-dispensing device, as small, e.g., less than 5,000, 1,000, 500, 250, 150, 100, 50, or 30 picoliter aliquots. In a preferred embodiment, the aliquots have imbibed into the continuous porous matrix.

The invention features the creation, by the use of piezo-electric dispensing, of two-dimensional arrays of probe molecules within a continuous three dimensional continuous porous matrix, e.g., a polymer matrix which may be supported by either a solid or porous substrate. Piezo-electric dispensing is advantageous because a very small volume can be dispensed into each array position. Small volumes result in small spot sizes in the two dimensional array (as projected onto a plane parallel to the support of the porous support matrix), since the dispensed volume occupies a very small volume when imbibed into the porous matrix. Small spot sizes are essential to achieve high densities of probes in the two dimensional array.

In preferred embodiments of the invention, molecules are dispensed into the matrix and become immobilized, preferably by covalent reaction, within the matrix. Preferably, chemical means are used to effect this immobilization while substantially preventing the migration of the probe molecules from the matrix or within the matrix. There are a variety of chemical means to accomplish this, e.g., described in the publication "Immobilization of DNA in Polyacrylamide Gel for the Manufacture of DNA and DNA-Oligonucleotide Microchips" by Proudnikov, et al., 1998, Analytical Biochemistry 259:34-41.

Embodiments provide an order of magnitude enhancement in signal compared to traditional planar surfaces because the three dimensional matrix provides a greater prove binding capacity while minimizing crowding and steric hindrances. In addition, arrays of the invention are protein and peptide applications because they provide three dimensional hydrophilic reaction environment.

Embodiments of the invention combine dispensing onto a polymer matrix with a piezo-electric dispenser, and the use physical and chemical means in this context to effect permanent immobilization of dispensed molecules within small, discrete spots, imbibed in the polymer matrix.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description Of The Drawings

Fig. 1 is a block diagram of the microvolume liquid handling system embodying the present invention;

Fig. 2 is a schematic of a positive displacement pump;

Fig. 3 is an illustration of a microdispenser and a piezoelectric transducer; and

Fig. 4 is a graph depicting the system line pressure during a microdispenser dispense.

Fig. 5 is a schematic view of two halves of a microtitre plate prior to being joined.

Fig. 6 is a sectional side plan view showing the two halves of the microtitre plate after having been joined .

Fig. 7 is a block diagram of a microvolume handling system.

Fig. 8 is an autograph showing the distribution of ^{33}P labeled oligonucleotides on an acrylamide slab which was dried and not processed further after dispensing 20 droplets per spot.

Fig. 9 is an autograph showing the distribution of ^{33}P labeled oligonucleotides dispensed onto a continuous polyacrylamide matrix with a piezoelectric dispenser and then processed with pyridine-borane complex under chloroform to covalently immobilize the oligonucleotides within the polyacrylamide matrix.

Fig. 10 is a storage phosphor image of spots of a ^{33}P labeled oligonucleotide dispensed onto a continuous polyacrylamide matrix with a piezoelectric dispenser and then washed with sodium borohydride rather than incubating the matrix with a reducing agent under chloroform.

Fig. 11A is a graph depicting the fluorescent intensity against the distance moved (distribution) by a probe in a gel matrix and activated glass. Fig. 11B is an autograph which depicts the corresponding fluorescent labeled probes.

Fig. 12A is a graph depicting the distribution of six fluorescent probes in a gel matrix. Fig. 12B is an autograph which depicts the corresponding fluorescent probes.

Fig. 13 is a graph depicting the net fluorescence (dNTP incorporated) with a polymer matrix, a glass surface activated by either a diisolthiocyanate, or a glass surface activated with an aldehyde group.

Fig. 14 A is a graph showing the distribution of three fluorescent probes. Fig. 14B shows the corresponding fluorescent target DNA molecules.

Fig. 15 is a graph showing the graph depicting the net fluorescence of a DNA target that was ligated with a polymer matrix, a glass surface activated by either a diisolthiocyanate, or a glass surface activated with an aldehyde group.

Fig. 16A is a graph showing the distribution of four fluorescent probes. Figure 16B is an autograph showing the corresponding fluorescent labeled molecules.

Detailed Description

Embodiments of the invention are based on an array having a plurality of addresses located on or in a continuous porous matrix. A continuous porous matrix can include a polymer matrix or gel, e.g., a polyacylamide gel, a polysaccharide gel, or an agarose gel. The array of the invention can be used to analyze a sequence, e.g., to sequence a test nucleic acid, or to identify SNPs, mutations and RNA molecules, or to clarify a sample, e.g., as to disease state, or generally in expression profiling or analysis. The analyses can be performed using circular vectors (as described in U.S. Patent No. 5,714,320). The circular vectors can be closed circular vectors, open circular vectors which when brought into contact with the analyte, have abutting ends which can be covalently linked, e.g., ligated.

Construction of Continuous Porous Matrix arrays

A continuous porous matrix should be a polymer matrix, e.g., a gel matrix, e.g., a hydrogel. Suitable material include polyacrylamide, agarose, dextran, or other polysaccharides. The area or volume occupied by a unique reagent at an address should be continuous, as opposed to being separate unconnected chambers or pores. In a preferred embodiment, the volume occupied by a plurality of addresses is continuous, as opposed to being separate unconnected chambers or pores. In general, any methods known in the art that are capable of generating arrays having postionally distinguishable molecules can be adapted for use in the methods described herein. Continuous porous matrix are preferably constructed by using a microvolume liquid handling system, e.g., a piezo-dispensing device, e.g., the device described in USSN 08/656,455, USSN 09/012,174 and 09/097,211, the contents of which are incorporated by reference.

Piezo-electric dispensing allows for a small volume, e.g., 350 picoliters, to be dispensed in or on the continuous porous matrix. Small volumes result in small spot sizes in the two dimensional arrays as projected onto a plane parallel to the support of the porous matrix, because the dispensed sample occupies a very small volume when applied to the porous matrix. Small spot sizes are important for achieving a high density of molecules, e.g., probe molecules, in the continuous porous array.

The two dimensional array may be disposed within a porous three-dimensional matrix, which is preferably a polymer matrix that is supported by either a solid or porous substrate.

Any chemistry that results in the immobilization of the unique reagent, e.g., the capture probe (e.g., a DNA, RNA, protein, or carbohydrate) within the continous porous polymer matrix and that prevents significant diffusion out of or within the matrix before immobilization occurs would be suitable for creating high density arrays of probe

molecules in three dimensional matrices by piezo-electric dispensing. Other suitable chemistries include the following. Chemistries described by the Mirzabekov group (e.g., "Regioselective immobilization of short oligonucleotides to acrylic copolymer gels", Timofeev, et al., 1996, Nucleic Acid Research 24:3142-3148) such as the reaction of aldehyde containing-probe molecules to hydrazide-containing matrices or the reaction of amine-containing probe molecules with glutaraldehyde-activated matrices. Some means to contain the molecules within the matrix while permitting hydration of the matrix is required to permit covalent immobilization while preventing diffusion of the molecules out of the matrix. This as been effected by the the use of chloroform or oil overlays ("DNA analysis and diagnostics on oligonucleotide microchips", Yershov, et al., 1996, Proc. Natl. Acad. Sci. 93:4913-4918).

Alternatively, molecules can be immobilized to the exterior surface of a gel particle using bifunctional reagents (e.g., see U.S. 4, 822, 747). In one example, a disulfide can be incorporated into the gel to immobilize capture probes which contain a moiety which reacts with the disulfide or sulfhydryl groups in the matrix, e.g., the capture probe can contain a thiol, e.g., see US 4,898,824.

Reaction of active esters such as N-Hydroxy-succinimidyl esters or isothiocyanates on probe molecules with amine-containing matrices. In some cases, such reactions will be fast enough to prevent diffusion of the probe molecules from or within the matrix before immobilization occurs, and overlay with immiscible liquids may no be required.

Reaction of photoactivatable groups on probe molecules (or on the matrix) with reactive groups on the matrix (or on the probe molecule). For example psoralen can be incorporated into the matrix, and nucleic acid probe molecules then react when the matrix was exposed to intense ultraviolet light. An focused beam of ultraviolet light could be

focused on the matrix immediately after dispensing the oligonucleotide to effect covalent immobilization before the probe molecules could diffuse significantly in the matrix.

Ligand molecules such as streptavidin could be incorporated into the matrix and probe molecules containing biotin could be dispense onto the matrix. This can immediately result in the stable, but non-covalent attachment of the probe molecules to the matrix.

In some embodiments, the support of the polymer matrix is made of a porous material which could allow the passage of electrical current to effect transport of molecules into our out of the matrix.

Positional arrays suitable for the present invention include high and low density arrays made with a continuous porous matrix. Positional arrays include nucleic acid molecules, peptide nucleic acids or high affinity binding molecules of known sequence attached to predefined locations on a surface. Arrays described in numerous patents which are incorporated herein by reference, Cantor, US Patent No.,5,503,980; Southern EP 0373 203 B1; Southern, U.S. Patent No. 5,700,637, and Deugau, U.S. Patent No. 5,508,169, can be adapted to continuous porous matrix arrays of the invention. The density of the array can range from a low density format a 96- or 384- array matrix, to a high density format, e.g. 1,000 molecules/cm2, as described in Fodor US 5,445,934.

In preferred embodiments, the target or probes bind to (and can be eluted from) the array at a single temperature. This can be effected by manipulating the length or concentration of the array or nucleic acid which hybridizes to it, by manipulating ionic strength or by providing modified bases.

Unique reagents, e.g., nucleic acid or polypeptide based polymers can be synthesized in situ or deposited preformed or in the array.

Proximity Methods

Proximity methods include those methods whereby a signal is generated when a first member and second member of a proximity detection pair are brought into close proximity can be used to evaluate the interaction of a sample with a reagent.

A "proximity detection pair" will have two members, the first member, e.g., an energy absorbing donor or a photosensitive molecule and the second member, e.g., an energy absorbing acceptor or a chemiluminescer particle. When the first and second members of the proximity detection pair are brought into close proximity, a signal is generated. Examples of proximity methods include the following:

Fluorescence resonance energy transfer (FRET)

Fluorescence resonance energy transfer (FRET) is based on a donor fluorophore that absorbs a photon of energy and enters an excited state. The donor fluorophore transfers its energy to an acceptor fluorophore when the two fluorophores are in close proximity by a process of non-radiative energy transfer. The acceptor fluorophore enters an excited state and eliminates the energy via radiative or non-radiative processes. Transfer of energy from the donor fluorophore to acceptor fluorophore only occurs if the two fluorophores are in close proximity.

Homogeneous time resolved fluorescence (HTRF)

Homogeneous time resolved fluorescence (HTRF) uses FRET between two fluorophores and measures the fluorescent signals from a homogenous assay in which all components of the assay are present during measurement. The fluorescent signal from HTRF is measured after a time delay, thereby eliminating interfering signals. One example of the donor and acceptor fluorophores in HTRF include europium cryptate [(Eu)K] and XL665, respectively.

In a preferred embodiment, the capture probes are molecular beacons. In this technology, a nucleic acid can exist in either of two forms, a hairpin, or other self annealed form, and in a form annealed to a second nucleic acid. In the self annealed configuration, a fluorophore and a quencher are in close proximity and no or comparatively little signal is emitted. When hybridized to a second nucleic acid , the flurophore and quencher are sufficiently separated that a greater level of signal is generated.

Luminescent oxygen channeling assay (LOCI)

In the luminescent oxygen channeling assay (LOCI), the proximity detection pairs includes a first member which is a sensitizer particle that contains phthalocyanine. The phthalocyanine absorbs energy at 680 nm and produces singlet oxygen. The second member is a chemiluminescer particle that contains olefin which reacts with the singlet oxygen to produce chemiluminescence which decays in one second and is measured at 570 nm. The reaction with the singlet oxygen and the subsequent emission depends on the proximity of the first and second members of the proximity detection pair.

Rolling Circle Amplification

Rolling circle can be used in some of the methods described herein. Rolling circle amplification (RCA) is used to generate many copies of an oligonucleotide preferably with defined ends (as described in U.S. Pat. No. 5,714,320). The single-stranded product of rolling circle amplification can be rendered double-stranded by the annealing of un-circularized, complementary probe vector. The dsDNA RCA product can be fragmented, e.g., using a type IIS restriction enzyme, such that the DNA is cleaved in the middle, or at the ends, of the region generated by the ligation reaction.

Rolling circle amplification products can be analyzed in situ or otherwise, e.g., on a second array, e.g., an array of indexing linkers (see, e.g., U.S. Patent No. 5,508,169). If the probe vector is labeled with an anchoring moiety, e.g., a biotin group, then it is possible to render the product generated from fragmentation of the RCA product single-stranded by thermal denaturation following the addition of capture or anchoring moiety reactive, e.g., strepavidin-labeled, substrates, e.g., magnetic beads or a solid support. The single-stranded DNA fragments can be analyzed on a Cantor array. These oligonucleotides are analyzed on a Cantor Capture array (see, e.g., 5,503,980) using, e.g., fluorescent detection methodology.

In other embodiments, the captured DNA fragments are analyzed using mass spectrometry. Alternatively, the target DNA is applied to a multiplicity of wells and a population of RCA vectors is added to each well. The RCA products are analyzed using mass spectrometry following fragmentation, where the amplification of specific RCA vectors is determined by differences in molecular weight of the RCA product fragments. Multiple RCA vectors can be analyzed simultaneously in a single reaction using this approach.

Methods of U.S. Patent No. 5,503,980 and or U.S. Patent No. 5,631,134, both of which are hereby incorporated by reference can be used herein, particularly, the array and array-related steps recited herein can use methods taught in these patents.

Rolling Circle and Additional Amplification

Although RCA (rolling circle amplification) in combination with very sensitive detection or additional round of RCA of signal amplification will often produce measurable signals without amplification, PCR (or some alternative like NASBA) may be desirable for achieving specific detection in some cases, e.g., in some cases of an allele in genomic DNA. Thus, regions of genomic DNA containing sites of polymorphisms can

be amplified by PCR prior to contact with circular templates. After PCR the unincorporated primers and dNTPs can be destroyed enzymatically (exonuclease and shrimp alkaline phosphates). The enzymes would then be destroyed by heating at 80? C.

PCR in Continuous porous matrix arrays and RCA Probes for Polymorphism Detection

SNP analysis of a large number of polymorphisms in a biallelic SNP map will sometimes require a relatively large number of amplification reactions. Amplification, e.g., PCR (or NASBA) can be performed continuous porous arrays with RCA probes. In this case the probe is annealed to the immobilized amplification product in the gel.

Probes for both (all four) alleles for all polymorphism sites on the array are applied to the array. The probes contain allele-specific tags, of which there are a total of only four - one for each base A, C, G, T. Presence of a restriction site is not necessary. In fact, it is unnecessary, since small fragments could diffuse from the gel arrays.

Only non-fluorescent dNTPs are present during the RCA reaction. The RCA products are labeled with generic allele-specific hybridization probes labeled with different color fluorophors, of which there are four (A, C, G, T). The sequences of the allele-specific tags and the probes obviously can be designed to provide very unambiguous differentiation of the four possible alleles, provided care is taken that the four fluorescent dyes are adequately separated. In this case there is great flexibility in the labeling of the probes (compared to the use of fluorescent ddNTP terminators).

Identification of RNA (RNA Profiling) and Sequencing of Mutations and SNPs Using Rolling Circle Amplification and Cantor Capture Arrays

A pre-formed circular vector can be applied to single-stranded cDNA in order to identify and quantitate the RNA molecules in a population of RNA molecules obtained from normal and disease cells. A population of circular vectors is applied to continuous gel arrays containing cDNA or RNA. The circular vectors can include:

(1) A region of random DNA sequence (e.g., 5-50 bases, preferably 12 bases);

(2) A region containing a recognition sequence for a type IIS restriction enzyme that cleaves in the middle of the region of random DNA sequence (note: this region may be designed to form a hairpin or other structure as described in 5,714,320);

(3) Additional DNA sequence that is, ideally, not complementary to any of the target nucleic acid sequences (RNA or cDNA) such that the complete vector contains between 50-1500 bases.

Those circular vectors that recognize sequences in the target are separated from the population of circular vectors added to the target nucleic acids. Background hybridization can be minimized by including linear DNA that contains all of the vector sequence except for the region of random DNA. The isolated circular vectors are amplified using rolling circle amplification (e.g., in the presence of a fluorescent nucleotides), the DNA is cleaving, e.g., using a restriction enzyme, and the resulting fragments are analyzed, e.g., interrogated on an indexing linker array (if dsDNA).

In another embodiment, circular vectors (as above) are used to identify the presence of mutations and SNPs by having a region of the circular DNA complementary to a mutation or SNP such that the circular DNA specifically binds to the mutation or SNP. Circular vectors complementary to a mutation or SNP will be isolated through application to a population of target DNA molecules (cDNA or RNA) e.g., bound to a continuous porous matrix array. The target DNA can be present as either an ordered

array of distinct molecules, or as a non-ordered array of molecules on a continuous porous matrix array. The resulting vectors are amplified by rolling circle amplification (e.g., in the presence of a fluorescent nucleotides), can be fragmented by restriction enzymes, and are analyzed, e.g., on an Indexing Linker (if dsDNA) see, e.g., 5,508,169 or a Cantor array (if ssDNA) see, e.g., 5,503,980.

Vectors can be separated into pools to prevent hybridization between the vectors (dsDNA probes should be avoided) and to maximize hybridization fidelity in any method described herein. The vector pools are applied to anchored target nucleic acid (genomic DNA, amplified DNA, cDNA or RNA) and those that hybridize to sequences in the target nucleic acid are isolated from the pool (conditions selected that maximize hybridization fidelity for each vector pool). The identity of the isolated vectors is determined by RCA, where the isolated oligo probes act as both a "positioning oligo" and an RCA primer (see 5,714,320). The DNA derived from rolling circle amplification (in the presence of a fluorescent nucleotides) is cleaved using a restriction enzyme, and the resulting fragments can be interrogated on an Indexing Linker array (if dsDNA) see, e.g., U.S. Patent No. 5,508,169 or a Cantor array (if ssDNA) see. e.g., U.S. Patent No.5,503,980.

DNA Sequencing

A linear DNA vector probe is designed with two, random, e.g., 5mer, sequences in either end of the vector. There are 1024 possible 5mer sequences, so this would entail the synthesis of 1,048,576 linear vectors. The vectors can share one or a small number of common backbones, where each backbone can include a type IIS restriction site and a priming site for DNA synthesis. The vectors can be grouped such that the random 5mers in a given group of vectors can not be brought together by the common backbone sequence. The sequence of the target nucleic acid will then facilitate the circularization of a subset of the probe vectors, with each circularized probe vector representing a short

contiguous, e.g., 10 bp, stretch of target DNA. The DNA is amplified using RCA in the presence of fluorescent nucleotides. The single-stranded product of rolling circle amplification is rendered double-stranded by the annealing of un-circularized, complementary probe vector. The dsDNA RCA product is analyzed. It can be fragmented, e.g., using a type IIS restriction enzyme such that the DNA is cleaved in the middle of the short region generated by the ligation reaction. The dsDNA fragments generated by the restriction digest are analyzed, e.g., on an array of indexing linkers (see, e.g., U.S. Patent No. 5,508,169). If the probe vector is labeled with a capture moiety, e.g., biotin group, then it is possible to render the dsDNA fragments generated from fragmentation of the RCA product single-stranded by thermal denaturation following the addition of capture moiety reactive, e.g., substrate, e.g., strepavidin-labeled substrate, e.g., magnetic beads or solid support. The single-stranded DNA fragments can then be analyzed on a Cantor array. The DNA sequence of the target DNA is reconstructed using overlap analysis according to the procedure of Drmanac et al. (see, e.g., 5,525,464; 5,492,806; 5,202,231; 5,695,940).

Dispensing Systems

Turning now to the drawings and referring first to FIG. 1, a microvolume liquid handling system 10 is illustrated. The microvolume liquid handling system 10 includes a positive displacement pump 12, a pressure sensor 14 and a microdispenser 16. Tubing 18 connects the positive displacement pump 12 to the pressure sensor 14 and the pressure sensor 14 to the microdispenser 16. The positive displacement pump 12 moves a system liquid 20 through the pressure sensor 14 and the microdispenser 16. After the system 10 is loaded with system liquid 20, an air gap 22 of known volume, then an amount of transfer liquid 24, are drawn into the microdispenser 16 in a manner described below. The transfer liquid 24 contains one or more reagents of interest. In one preferred embodiment the

microdispenser 16 expels (or synonymously, "shoots") sub-nanoliter size individual droplets 26 which are very reproducible. The expelled droplets 26 of transfer liquid 24 are on the order of 0.45 nanoliters per droplet 26 in one preferred embodiment, but they can be as small as 5 picoliters. For example, if one desires to expel a total of 9 nanoliters of transfer liquid 24, then the microdispenser 16 will be directed to expel 20 droplets 26. Droplet 26 size can be varied by varying the magnitude and duration of the electrical signal applied to the microdispenser 16. Other factors affecting droplet size include: the size of the nozzle opening at the bottom of the microdispenser, the pressure at the microdispenser inlet, and properties of the transfer liquid.

Referring now to FIGS. 1 and 2, in one preferred embodiment the positive displacement pump 12 is a XL 3000 Modular Digital Pump manufactured by Cavro Scientific Instruments, Inc., 242 Humboldt Court, Sunnyvale, California 94089. The positive displacement pump 12 includes stepper motor 28 and stepper motor 29, and a syringe 30. The syringe 30 includes a borosilicate glass tube 32 and a plunger 34, which is mechanically coupled through a series of gears and a belt (not shown) to the stepper motor 28. Stepper motor 28 motion causes the plunger 34 to move up or down by a specified number of discrete steps inside the glass tube 32. The plunger 34 forms a fluid tight seal with the glass tube 32. In one preferred embodiment syringe 30 has a usable capacity of 250 microliters which is the amount of system liquid 20 the plunger 34 can displace in one full stroke. Depending on the selected mode of operation, the stepper motor 28 is capable of making 3,000 or 12,000 discrete steps per plunger 34 full stroke. In one preferred embodiment the stepper motor 28 is directed to make 12,000 steps per full plunger 34 stroke with each step displacing approximately 20.83 nanoliters of system liquid 20. In one preferred embodiment the system liquid 20 utilized is deionized water.

Digitally encoded commands cause the stepper motor 28 within the positive displacement pump 12 to aspirate discrete volumes of liquid into the microdispenser 16,

wash the microdispenser 16 between liquid transfers, and to control the pressure in the system liquid 20 line for microvolume liquid handling system 10 operation. The positive displacement pump 12 is also used to prime the system 10 with system liquid 20 and to dispense higher volumes of liquid through the microdispenser 16, allowing dilute solutions to be made. The positive displacement pump 12 can also work directly with transfer liquid 24. Thus, if desired, transfer liquid 24 can be used as system liquid 20 throughout the microvolume liquid handling system 10.

To prime the microvolume liquid handling system 10, the control logic 42 first directs a 3-axis robotic system 58 through electrical wire 56 to position the microdispenser 16 over a wash station contained on the robotic system 58. In one preferred embodiment the microvolume liquid handling system 10 includes, and is mounted on, a 3-axis robotic system is a MultiPROBE CR10100, manufactured by Packard Instrument Company, Downers Grove, Illinois. The positive displacement pump 12 includes a valve 38 for connecting a system liquid reservoir 40 to the syringe 30. An initialization control signal is transmitted through the electrical cable 36 to the pump 12 by control logic 42 which causes the valve 38 to rotate connecting the syringe 30 with the system fluid reservoir 40. The control signal also causes the stepper motor 28 to move the plunger 34 to its maximum extent up (Position 1 in FIG. 2) into the borosilicate glass tube 32. The next command from the control logic 42 causes the stepper motor 28 to move the plunger 34 to its maximum extent down (Position 2 in FIG. 2) inside the tube 32, to extract system liquid 20 from the system reservoir 40. Another command from the control logic 42 directs the valve 38 to rotate again, causing the syringe 30 to be connected with the tubing 18 connected to the pressure sensor 14. In one preferred embodiment the tubing 18 employed in the microvolume liquid handling system 10 is Natural Color Teflon Tubing made by Zeus Industrial Products, Inc., Raritan, New Jersey, with an inner diameter of 0.059 inches and an outer diameter of 0.098 inches.

The next command from the control logic 42 to the positive displacement pump 12 causes the system liquid 20 inside of the syringe 30 to be pushed into the microvolume liquid handling system 10 towards the pressure sensor 14. Because the microvolume liquid handling system 10 typically requires about 4 milliliters of system fluid to be primed, the sequence of steps described above must be repeated about 16 times in order to completely prime the microvolume liquid handling system 10.

The control logic 42 receives signals from the pressure sensor 14 through an electrical line 46. The signals are converted from an analog form into a digital form by an A/D (analog to digital) converter 44 and used by the control logic 42 for processing and analysis. In one preferred embodiment the A/D conversion is a PC-LPM-16 Multifunction I/O Board manufactured by National Instruments Corporation, Austin, Texas. At various points in the liquid transfer process described herein, the control logic 42 receives signals from the pressure transducer 14, and sends command signals to the pump 12, microdispenser electronics 51, and the 3-axis robotic system 58. Within the control logic 42 are the encoded algorithms that sequence the hardware (robotic system 58, pump 12,and microdispenser electronics 51) for specified liquid transfer protocols as described herein. Also within the control logic 42 are the encoded algorithms that process the measured pressure signals to: verify and quantify microdispenses, perform diagnostics on the state of the microvolume liquid handling system, and automatically perform a calibration of the microdispenser for any selected transfer liquid 24.

The pressure sensor 14 senses fluctuations in pressure associated with priming the microvolume liquid handling system 10, aspirating transfer liquid 24 with pump 12, dispensing droplets 26 with microdispenser 16, and washing of microdispenser 16 using pump 12. In one preferred embodiment the pressure sensor 14 is a piezoresistive pressure sensor part number 26PCDFG6G, from Microswitch, Inc., a Division of Honeywell, Inc., 11 West Spring Street, Freeport, Illinois 61032. Also included with the

pressure sensor 14 in the block diagram in Figure 1 is electrical circuitry to amplify the analog pressure signal from the pressure sensor. The pressure sensor 14 converts pressure into electrical signals which are driven to the A/D converter 44 and then used by the control logic 42. For example, when the microvolume liquid handling system 10 is being primed, the pressure sensor 14 will send electrical signals which will be analyzed by the control logic 42 to determine whether they indicate any problems within the system such as partial or complete blockage in the microdispenser 16.

Once the microvolume liquid handling system 10 is primed, the control logic 42 sends a signal through electrical wire 56 which instructs the robotic system 58 to position the microdispenser 16 in air over the transfer liquid 24. The control logic 42 instructs stepper motor 28 to move the plunger 34 down, aspirating a discrete quantity of air (air gap), e.g., 50 microliters in volume into the microdispenser 16. The control logic 42 then instructs the robotic system 58 to move the microdispenser 16 down until it makes contact with the surface of the transfer liquid 24 (not shown) is made. Contact of the microdispenser 16 with the surface of the transfer liquid 24 is determined by a capacitive liquid level sense system (U.S Patent Number 5,365,783). The microdispenser is connected by electrical wire 55 to the liquid level sense electronics 54. When the liquid level sense electronics 54 detects microdispenser 16 contact with transfer liquid 24 surface, a signal is sent to the robotic system 58 through electrical wire 53 to stop downward motion.

The control logic 42 next instructs the pump 12 to move the plunger 34 down in order to aspirate transfer liquid 24 into the microdispenser 16. The pressure signal is monitored by control logic 42 during the aspiration to ensure that the transfer liquid 24 is being successfully drawn into the microdispenser 16. If a problem is detected, such as an abnormal drop in pressure due to partial or total blockage of the microdispenser, the control logic 24 will send a stop movement command to the pump 12. The control logic 24 will

then proceed with an encoded recovery algorithm. Note that transfer liquid 24 can be drawn into the microvolume liquid handling system 10 up to the pressure sensor 14 without threat of contaminating the pressure sensor 14. Additional tubing can be added to increase transfer liquid 24 capacity. Once the transfer liquid 24 has been aspirated into the microdispenser 16, the control logic 42 instructs the robotic system 58 to reposition the microdispenser 16 above the continuous porous matrix, e.g., a polymer matrix or gel., an acrylamide gel, a polysaccharide gel, or an agarose gel.

In one preferred embodiment the microdispenser 16 is the MD-K-130 Microdispenser Head manufactured by Microdrop, GmbH, Muhlenweg 143, D-22844 Norderstedt, Germany.

As illustrated in FIG. 3, the microdispenser 16 consists of a piezoceramic tube 60 bonded to a glass capillary 62. The piezoceramic tube has an inner electrode 66 and an outer electrode 68 for receiving analog voltage pulses which cause the piezoceramic tube to constrict. Once the glass capillary 62 has been filled with transfer liquid 24, the control logic 42 directs the microdispenser electronics 51 by electrical wire 50 to send analog voltage pulses to the piezoelectric transducer 60 by electrical wire 52. In one preferred embodiment the microdispenser electronics 51 is the MD-E-201 Drive Electronics manufactured by Microdrop, GmbH, Muhlenweg 143, D-22844 Norderstedt, Germany. The microdispenser electronics 51 control the magnitude and duration of the analog voltage pulses, and also the frequency at which the pulses are sent to the microdispenser 16. Each voltage pulse causes a constriction of the piezoelectric transducer 60, which in turn deforms the glass capillary 62. The deformation of the glass capillary 62 produces a pressure wave that propagates through the transfer liquid 24 to the microdispenser nozzle 63 where one droplet 26 of transfer liquid 24 is emitted under very high acceleration. The size of these droplets 26 has been shown to be very reproducible. The high acceleration of the transfer liquid 24 minimizes or eliminates problems caused by transfer liquid 24 surface tension and

viscosity, allowing extremely small droplets 26 to be expelled from the nozzle, e.g., as small as 5 picoliter droplets 26 have been demonstrated. Use of the microdispenser 16 to propel droplets 26 out of the nozzle also avoids problems encountered in a liquid transfer technique called touchoff. In the touchoff technique, a droplet 26 is held at the end of the nozzle and is deposited onto a continuous porous matrix by bringing that droplet 26 into contact with the continuous porous matrix while it is still hanging off of the microdispenser 16. Such a contact process is made difficult by the surface tension, viscosity and wetting properties of the microdispenser 16 and the target surface which lead to unacceptable volume deviations. The present invention avoids the problems of the contact process because the droplets 26 are expelled out of the microdispenser 16 at a velocity of several meters per second. The total desired volume is dispensed by the present invention by specifying the number of droplets 26 to be expelled. Because thousands of droplets 26 can be emitted per second from the microdispenser 16, the desired microvolume of transfer liquid 24 can rapidly be dispensed.

In one preferred embodiment, the lower section of the glass capillary 62, between the piezoelectric transducer 60 and the nozzle 63, is plated with a conductive material, either platinum or gold. This provides an electrically conductive path between the microdispenser 16 and the liquid level sense electronics 54. In one preferred embodiment the glass capillary 62 has an overall length of 73 millimeters, and the nozzle 63 has an internal diameter of 75 micrometers.

To dispense microvolume quantities of transfer liquid 24, analog voltage pulses are sent to the microdispenser 16, emitting droplets 26 of liquid. Capillary forces acting on the transfer liquid 24 replace the volume of transfer liquid 24 emitted from the microdispenser 16 with liquid from the tubing 18. However, since the transfer liquid-air gap-system liquid column terminates at a closed end in the positive displacement pump 12, there is a corresponding drop in the system liquid 20 line pressure as the air gap 22 is

expanded. This is illustrated in Figure 4 which depicts the pressure profile measured during a microdispense of 500 nanoliters. Important to the present invention, the magnitude of the pressure drop is a function of the size of the air gap 22 and the volume of the liquid dispensed.

With an air gap 22 of known volume, the pressure change as detected by the pressure sensor 14 relates to the volume dispensed. Thus, the control logic 42 determines from the pressure change measured by the pressure sensor 14, the volume of transfer liquid 24 that was dispensed. In one preferred embodiment of the present invention it is preferable that the drop in pressure not exceed approximately 30 to 40 millibars below ambient pressure, depending on the properties of the transfer liquid 24. If the amount of transfer liquid 24 dispensed is sufficient to drop the pressure more than 30 to 40 millibars, the pressure difference across the microdispenser 16, i.e., between the ambient pressure acting on the nozzle 63 and the pressure at the capillary inlet 63, will be sufficient to force the transfer liquid 24 up into the tubing 18. This will preclude further dispensing. There is a maximum amount of transfer liquid 24 that can be dispensed before the control logic 42 is required to command the pump 12 to advance the plunger 34 to compensate for the pressure drop. This maximum volume is determined by the desired dispense volume and the size of the air gap 22. Conversely, the size of the air gap 22 can be selected based on the desired dispense volume so as not to produce a pressure drop exceeding 30 to 40 millibars below ambient pressure. It is also within the scope of the present invention to advance the plunger 34 while the microdispenser 16 is dispensing, thereby rebuilding system liquid 20 line pressure, so that the microdispenser 16 can operate continuously.

The change in system liquid 20 pressure is used to determine that the desired amount of transfer liquid 24 was dispensed. A second verification of the amount of transfer liquid 24 that was dispensed is made by the control logic 42 monitoring the system

liquid 20 line pressure while directing the pump 12 to advance the syringe plunger 34 upwards towards Position 1. The syringe plunger 34 is advanced until the system liquid 20 line pressure returns to the initial (pre-dispense) value. By the control logic 42 tracking the displaced volume the plunger 34 moves (20.83 nanoliters per stepper motor 28 step), a second confirmation of dispensed volume is made, adding robustness to the system. The system liquid 20 line pressure is now at the correct value for the next microdispenser 16 dispense, if a multi-dispense sequence has been specified.

Once the transfer liquid 24 dispensing has been completed, the control logic 24 causes the robotic system 58 to position the microdispenser 16 over the wash station. The control logic 24 then directs pump 12 and robotic system 58 in a wash sequence that disposes of any transfer liquid 24 left in the microdispenser 16, and washes the internal surface of glass capillary 62 and the external surface in the nozzle 63 area that was exposed to transfer liquid 24. The wash fluid can either be system liquid 20 or any other liquid placed onto the deck of the robotic system 58. The wash sequence is designed to minimize cross-contamination of subsequent transfer liquids 24 with transfer liquids processed prior. Toward this end, it is also possible to enable an ultrasonic wash of the microdispenser 16. This is accomplished by the control logic 42 directing the microdispenser electronics 51 to send electrical pulses to the microdispenser at a frequency in the ultrasonic range, e.g., 12 - 15 kilohertz, that coincides with a resonant frequency of the microdispenser 16 - transfer liquid 24 system.

Activating the piezoelectric transducer 60 at ultrasonic frequencies resonant with the glass capillary 62 of the microdispenser 16 causes the interior surfaces of the glass capillary 62 to vibrate vigorously. In both the first and second embodiments, system liquid 20 or a special cleaning and/or neutralizing fluid is used to flush out the microdispenser 16 while the piezoelectric transducer 60 is activated at resonant frequencies. Cleaning with resonant ultrasonic excitation has the effect of far more

efficiently dislodging and eliminating matter adhering to the microdispenser 16. For example, it has been shown in a number of test cases that ultrasonic excitation caused a 200% to 500% improvement (depending on the contaminant) in the reduction of residual matter left in the microdispenser 16 as compared to cleaning without ultrasonic excitation.

Resonant ultrasonic excitation of the microdispenser 16 also is used to prevent, minimize or alleviate clogging of the nozzle of the microdispenser. For example, when transfer liquid is being aspirated into the microdispenser 16 it must pas through the relatively narrow nozzle 63 in the glass capillary 62. Matter in the transfer liquid 24 often comes into contact with the nozzle's 63 surfaces permitting the matter to adhere to the nozzle 63, depending on the nature of the contact. In biochemical applications, one widely used matter added to the transfer liquid 24 is polystyrene spheres. The spheres typically range from 1 μM to over 30 μM and may be uncoated or coated with magnetic ferrites, antigens or other materials. The relatively large size of the polystyrene spheres with regard to nozzle 63 diameter, in combination with their sometimes sticky coatings, can cause the spheres to adhere to the nozzle 63. It has been discovered that if the piezoelectric transducer 60 is excited at the ultrasonic resonant frequency of the microdispenser 16 while the microdispenser 16 is being loaded (i.e. transfer liquid 24 is being aspirated in the microdispenser 160 that clogging is prevented or less likely to occur. Thus, ultrasonic excitation of the microdispenser 16 works to prevent or diminish clogging of the nozzle 63 by materials in the transfer liquid 24.

Anytime a transfer liquid 24 containing dissolved or suspended materials passes through the nozzle 63 there is possibility of clogging. Accordingly, not only is clogging a problem during aspiration of transfer liquid 24 into the microdispenser 16 as described above, but it is also a problem when transfer liquid is dispensed from the microdispenser 16. It has been discovered that periodic resonant ultrasonic excitation of the

microdispenser 16 between droplet dispensing by the piezoelectric transducer can reduce buildup of materials adhering to the nozzle 63 and thus prevent clogging in some instances. Even if substantial clogging does occur, resonant ultrasonic excitation of the microdispenser 16 by the piezoelectric transducer 60 will substantially clear the clogging materials from the nozzle 63, the microvolume liquid handling system 10 can continue operation without resort to extraordinary cleaning procedures and the delays associated with those procedures. In short, system downtime is reduced, thereby making the microvolume liquid handling system 10 more efficient.

In the above description of the invention, the control of the microdispenser 16 was effected by sending a specific number of electrical pulses from the microdispenser electronics 51, each producing an emitted droplet 26 of transfer liquid 24. It is also within the scope of the invention to control the microdispenser 16 by monitoring the pressure sensor 14 signal in realtime, and continuing to send electrical pulses to the microdispenser 16 until a desired change in pressure is reached. In this mode of operation, the PC-LPM-16 Multifunction I/O Board that contains the A/D converter 44 is instructed by control logic 42 to send electrical pulses to the microdispenser electronics 51. Each pulse sent by the Multifunction I/O Board results in one electrical pulse that is sent by the microdispenser electronics 51 to the microdispenser 16, emitting one droplet 26 of transfer liquid 24. The control logic 42 monitors the pressure sensor 14 signal as the microdispenser 16 dispense is in progress, and once the desired change is pressure has been attained, the control logic 42 directs the Multifunction I/O Board to stop sending electrical pulses.

This mode of operation is employed if a "misfiring" of microdispenser 16 has been detected by control logic 42.

It is also within the scope of the invention for the microvolume liquid handling system 10 to automatically determine (calibrate) the size of the emitted droplets 26 for transfer liquids 24 of varying properties. As heretofore mentioned, emitted droplet 26 size

is affected by the properties of the transfer liquid 24. Therefore, it is desirable to be able to automatically determine emitted droplet 26 size so that the user need only specify the total transfer volume, and the system 10 will internally determine the number of emitted droplets 26 required to satisfy the user request. In the encoded autocalibration algorithm, once the system 10 is primed, an air gap 22 and transfer liquid 24 aspirated, the control logic 42 instructs microdispenser electronics 51 to send a specific number of electrical pulses, e.g., 1,000, to the microdispenser 16. The resulting drop in pressure sensor 14 signal is used by control logic 42 to determine the volume of transfer liquid 24 that was dispensed. This dispensed volume determination is verified by the control logic 42 tracking the volume displaced by the movement of the plunger 34 to restore the system liquid 20 line pressure to the pre-dispense value.

The microvolume liquid handling system 10 illustrated is FIG. 1 depicts a single microdispenser 16, pressure sensor 14, and pump 12. It is within the spirit and scope of this invention to include embodiments of microvolume liquid handling systems that have a multiplicity (e.g., 4, 8, 96) of microdispensers 16, pressure sensors 14, and pumps 12. It is also within the spirit and scope of this invention to include embodiments of microvolume liquid handling systems that have a multiplicity of microdispensers 16, pressure sensors 14, valves 38, and one or more pumps 12.

The following description illustrates the use of a device with a microtitre plate. The plate can be adapted for use with the continuous porous matrix of the invention.

Turning now to FIGS. 5, 6 and 7, one application for drop-on-demand microvolume fluid dispensing is to deposit precise amounts of transfer liquid 24 into an array of wells in a microtitre plate 110, which is described in U.S. Patent No. 5,457,527, hereby incorporated by reference. The microtitre plate 110 is formed from two molded plastic plates 111 and 112. The upper plate 111 forms the side walls 113 of the multiple wells of the microtitre plate, and in the illustrative example, the wells are arranged in an 8

x 12 matrix, although matrices with other dimensions also work with the present invention. The bottom plate 112 forms the bottom walls 114 of the matrix web, and is attached to the lower surface of to the lower surface of the upper plate by fusing the two plates together. The upper plate 111 is formed from an opaque polymeric material so that light cannot be transmitted therethrough. In contract to the upper plate 111, the lower plate 112 is formed of a transparent polymeric material so that it forms a transparent bottom wall 114 for each sample well. This permits viewing of sample material through the bottom wall 114, and also permits light emissions to be measured through the bottom wall. The transparent bottom walls 114 may also be used to expose the sample of light from an external excitation source, while leaving the tops of the wells unobstructed for maximum detection area.

In part because the present microvolume fluid dispensing system 10 can precisely dispense extremely small quantities of fluid, it is possible to utilize microtitre arrays 110 of correspondingly reduced dimensions. The difficulty of positioning the nozzle 63 directly over each well increases as the well diameter approaches the one millimeter range. In the case of a well diameter of one millimeter, it is desirable to position the nozzle 63 within 150 micrometers ("μM") of the center of the well to permit accurate droplet shooting. The present invention utilizes a transparent bottom portion 112 of the microtitre plate array 110 which allows visible and infrared light to pass through the bottom of the microtitre array 110 into the well formed by the opaque side walls 113 of the microtitre plate array 111 and the transparent bottom walls 114 of the transparent bottom array 112. In one embodiment infrared light is passed through the transparent bottom section 112 of the microtitre plate array 110 onto the glass capillary 62 of the microdispenser 16. The light received at the microdispenser 16 is passed through the glass capillary 62 to an appropriate infrared detector (not shown) mounted on the glass capillary 62. The infrared light source, in combination with the narrow well structure,

provides a narrow beam of infrared light directed upward through each well, but not through an opaque material between the wells. As the microdispenser is moved from one well to another it encounters a relatively dark zone indicating the dispenser is between wells, followed by a relatively bright zone indicating the edge of the next well is directly below. The positioning robot then uses these cues to reach and verify the position of the microdispenser.

In another preferred embodiment, visible light is used in place of infrared light as described above. For example, any visible wavelength of light can be used if the wells are devoid of liquid, or have clear liquids and a matching detector is used in place of the infrared detector. In the case where a turbid or cloudy liquid is present in the wells, a greenish light at 300 nM can be passed through the microtitre plate 110 to the turbid liquid. A cryptate compound added to the liquid present in the well fluoresces in response to excitation by the greenish light. Cryptate fluoresces at approximately 620 and 650 nM, corresponding to red light. A detector that detects those red wavelengths is used in place of the infrared detector.

Turning now to FIG. 7, the second preferred embodiment of the microvolume liquid handling system 210 is shown. The second preferred embodiment is more preferred than the first preferred embodiment when the number of microdispensers 212 employed is equal to, or greater than, eight because the second embodiment becomes more cost effective as the number of microdispensers 212 is increased. When the number of microdispensers 212 employed is equal to or less than four, the first preferred embodiment is more preferred than the second preferred embodiment because the first embodiment becomes more cost effective when small numbers of microdispensers 212 are employed. The tradeoff occurs because in the second preferred embodiment a system liquid reservoir 214 is used to supply system liquid 20 to all the microdispensers 212, thus eliminating the separate pump and pressure sensor for each microdispenser 212 in

the first preferred embodiment. However, because the system liquid reservoir 214 is more expensive to implement, it is more cost effective to employ the first embodiment when four or fewer microdispensers are employed. Note that first and second preferred embodiments are otherwise identical in structure and operation except as described herein. The precise number of microdispensers employed is a function of the user's dispensing requirements.

With regard to the second preferred embodiment, the system liquid reservoir 214 receives system liquid 20, typically deionized water, through an intake tube 216 which contains a cap (not separately shown). The cap on the intake tube 216 is removed to enable the sealed system liquid reservoir 214 to receive system liquid 20 when the cap is off and seals the system liquid reservoir 214 shut when the cap is on so that the system liquid reservoir 214 can be maintained at a desired pressure. Pressure in the system liquid reservoir 214 is maintained by a pressure control system 218, through pressure control tubing 220. The pressure control system 218 includes an electrically controlled pump capable of accurately increasing or decreasing pressure in the system liquid reservoir 214. A pressure sensor 222 mounted on the system liquid reservoir 214 senses pressure in the system liquid reservoir 214 and transmits an electrical signal indicative of that pressure to a system controller 224 through electrical conductor 226. The system controller 224 contains a digital signal processor board and other electronics (not shown), which enable monitoring of various electrical signals, execution of control software code, and control of the microvolume liquid handling system 210. The system controller 224 electrically controls the pressure control system 218 through an electrical conductor 228 to adjust the pressure of the system liquid 20, and correspondingly, the pressure of the transfer liquid 24. A pressure relief valve 230 is mounted on the system liquid reservoir 214 when the pressure exceeds a predetermined safety threshold. In one embodiment the

pressure relief valve 230 can also be opened by the system controller 224, which is connected to the pressure relief valve 230 by a wire 232.

During operations, the system controller 224 directs the pressure control system 218 to maintain one of three different pressure levels in the system reservoir 214 with regard to ambient atmospheric pressure. Each of the three pressure levels correspond to a different phase of operation of the microvolume liquid handling system 210. The three different pressure levels are a positive pressure, a high negative pressure and a low negative pressure. Prior to dispensing, the positive pressure level is used for cleaning in order to flush the microdispenser free of any foreign matter in combination with resonant ultrasonic excitation of the microdispensers 212 in the manner described above. After the microdispensers 212 are relatively clean, the high negative pressure level, roughly 200 milibars less than the ambient atmospheric pressure, is used to aspirate transfer liquid 24 into the microdispensers 212. Once the transfer liquid 24 has been aspirated into the microdispensers 212, the low negative pressure level, roughly 15 milibars, is used to supply back pressure to the transfer liquid 24 in the microdispensers 212 such that as droplets are dispensed, no additional transfer liquid 24 leaves the microdispensers 212.

System liquid 20 in the system reservoir 214 is coupled to the microdispensers 212 through a distribution tube 234 that splits into a plurality of sections as shown in FIG.7, one section is connected to each microdispenser 212. Attached to each of the distribution tube sections 234 are microvalves 242 and flow sensors 244. The microvalves 242 are micro-electro-mechanical machines ("MEMS") that have the primary advantage of being sufficiently small so as to fit easily into the microvolume liquid handling system 210. The microvalves 242 are extremely precise valves used to control the movement of system liquid 20 and correspondingly, the amount of transfer liquid 24 that is dispensed. The system controller 224 sends electrical signals through an electrical connection 246 to control the microvalves 242. A flow sensor 244 is attached

to each distribution tube section 234 to determine the amount of liquid that is being aspirated into each microdispenser associated with that flow sensor 244. The flow sensor 244 detects flow of system liquid 20 into or out of each microdispenser 212. The flow sensors 244 are each connected to the system controller 224 through an electrical conductor 248. The electrical conductor 248 carries electrical signals from each flow sensor 244 indicating not only the amount of liquid flow, but also the pressure in the distribution tube 234. The flow sensors 244 are also MEMS that have the primary advantage of being sufficiently small so as to fit easily into the microvolume liquid handling system 210, for example the flow sensors 244 described in IEEE Proceedings, MEMS 1995, publication number 0-7803-2503-6, entitled, A Differential Pressure Liquid Flow Sensor For Flow Regulation and Dosing Systems, by M. Boillat et al.

The distribution tube 234, which is physically connected to the microdispensers 212, is attached to a three-axis robot 238, as in the first preferred embodiment, which correspondingly relocates the microdispensers 212 to positions above different microtitre plate 110 wells. After the desired number of droplets has been dispensed into each well, the robot 238 moves the microdispensers 212 to the next set of wells for further dispensing. Precise coordination of the robot's 238 movement is accomplished as described above with reference to the use of light passed through the bottom microtitre plate 112.

Example 1

Oligonucleotide probe molecules were attached to an polyacrylamide gel as described in Proudnikov et al., Anal. Biochem. 259:34-41, 1998; Timofeev et al., NAR 24:3142-48, 1996. Briefly, a continuous slab of polyacrylamide gel about 20μm thick was formed on a glass microscope slide by polymerization between the glass slide and a glass plate. The monomer N-(2,2dimtheyoxy)ethyl acrylamide was incorporated into the

polyacrylamide matrix during polymerization. Before oligonucleotides were dispensed, the gel matrix was activated by incubation with aqueous trifluroacetic acid to convert diol groups in the gel to aldehyde groups. The gel was then dried.

A total of 20 drops of amine-derivatized oligonucleotides labeled with ^{32}-P were dispensed onto the surface of the dried gel. The gel absorbs the liquid sample and then eventually dries with the sample trapped in the matrix. The distribution of the labeled oligonucleotide an a dried acrylamide slab was examined by phosphor imaging and found to consists of a series of discrete, resolved spots. Figure 8 shows the distribution of the ^{33}P labeled oligonucleotide on an acrylamide slab which was dried and not processed further after dispensing 20 droplets per spot.

Example 2

A ^{32}-P labeled oligonucleotide sample was applied using a piezo dispenser as described in Example 1. The continuous polyacrylamide matrix was placed under a layer of chloroform which, in turn, was overlaid with water containing a pyridine-borane complex. Water was able to diffuse through the chloroform layer to hydrate the gel, but the chloroform prevented diffusion of the oligonucleotide out of the gel. Schiff bases that formed between the amine groups on the oligonucleotides and the aldehyde groups with the gel were reduced by the pyridine-borane complex, which also diffused through the chloroform layer to covalently link the oligonucleotide probe molecules to the acrylamide matrix. Phosphor imaging of ^{32}-labeled oligonucleotides after reducing and washing the matrix revealed that the oligonucleotide remained in discrete spots, similar to the pattern observed on polyacrylamide substrates that was not processed after dispensing as described in Example 1. Quantitation revealed that 80 percent of the oligonucleotide was retained in the matrix (Figure 9).

Example 3

A ^{32}-labeled oligonucleotide was applied to a gel matrix as described in Example 1, except that the slide was simply washed with sodium borohydride, a reducing agent, after dispensing. The covalently immobilization step was also omitted. Under these conditions, approximately 80% of the oligonucleotide was washed out of the matrix. These results demonstrate that an linking step is required to retain the oligonucleotides within the gel matrix. Figure 10 is a storage phosphor image of spots of a ^{33}P labeled oligonucleotide dispensed onto a continuous polyacrylamide matrix with a piezoelectric dispenser and then washed with sodium borohydride rather than incubating the matrix with a reducing agent under chloroform.

Example 4

It will be seen from the following examples that despite relatively low coupling efficiency very effective quantities of probe molecules can be immobilized with the simple procedure used in Example 3.

An amine derivatized oligonucleotide probe was dispensed onto the surface of an activated (aldehyde containing) gel matrix as in Example 1, except that only a single droplet (350 picoliters) of the oligonucleotide solution (100 micromolar) was dispensed onto each position of the array. The slide containing the gel matrix was washed with sodium borohydride as in Example 3. This effected the reduction of Schiff bases and hence the covalent immobilization of a fraction of the oligonucleotide probe in the matrix. After immobilization, the array was hybridized with a fluorescently labeled target oligonucleotide (400 femptomoles in 50 microliters) that was complementary to the probe. After washing away unhybridized target oligonucleotide and drying of the matrix, the slide was scanned with a fluorescent scanner.

A portion of the resulting image is shown in Figure 11 with a profile (solid line) of the fluorescent intensity in the gel matrix to indicate the distribution of the fluorescent target in one dimension across three of the spots containing immobilized probe. It can be appreciated from Figure 11 that the target oligonucleotide hybridized specifically to the spots containing the immobilized probe, and that the probe was immobilized in discrete spots within the polymer matrix. The spots have a diameter of about 185 micrometers. Spot diameters as small as this can be achieved only by dispensing droplets of very small volume. For comparison, the profile of the fluorescent intensity is shown in Figure 11 from an experiment done exactly the same way except that the probe was dispensed onto a commercially available slide with an activated "polymeric coating" (dashed line). At the high target concentration used in this experiment, one observes a substantially greater binding capacity with the gel matrix.

Example 5

An oligonucleotide with an amine on the 5' end was dispensed onto the surface of an activated (aldehyde containing) gel as in Example 1, except that only a single droplet (350 picoliters) of the oligonucleotide solution (50 micromolar) was dispensed onto each position of the array. The slide containing the gel matrix was washed with sodium borohydride as in Example 3. This effected the reduction of Schiff bases and hence the covalent immobilization of a fraction of the oligonucleotide in the matrix by its 5' end, so that the 3' end was free in solution and available for extension by a template-directed polymerase reaction. After immobilization, the array was incubated with a target DNA molecule that was complementary and longer than the immobilized oligonucleotide primer, a polymerase and dideoxy nucleotide triphosphates (ddNTPs). The dideoxyguanosine nucleotide was fluorescently labeled and was expected to be

incorporated onto the 3' end of the immobilized oligonucleotide by the template-directed reaction, thus confirming the identity of a single base of the oligonucleotide target – a procedure commonly referred to as minisequencing. After washing away unincorporated nucleotides and drying of the matrix, the slide was scanned with a fluorescent scanner.

A portion of the resulting image is shown in Figure 12 with a profile of the fluorescent intensity to indicate the distribution of the fluorescent target in one dimension across six of the spots containing immobilized oligonucleotide. It can be appreciated from Figure 12 that the fluorescent nucleotide was incorporated into the spots containing the immobilized oligonucleotide, and that the oligonucleotide was immobilized in discrete spots, which are very uniform. The spots have a diameter of about 180 micrometers. Figure 13 shows a comparison of the relative amount of ddNTP incorporated when the experiment was performed with a polymer matrix substrate and when it was performed in exactly the same way except that the amine-derivatized oligonucleotide was immobilized on glass surfaces activated either with diisothiocyanate or with aldehyde groups. Results for two different oligonucleotide primers ("15T-S84" and "15T-S86") and their corresponding targets are shown. In both cases the nucleotide incorporation was substantially greater with the polymer matrix than the activated planar glass surfaces.

Example 6

A partially self-complementary oligonucleotide probe capable of forming a hairpin structure and having an amine on a internal nucleotide in the loop of the hairpin was dispensed onto the surface of an activated (aldehyde containing) gel as in Example 1, except that only a single droplet (350 picoliters) of the oligonucleotide solution (100

micromolar) was dispensed onto each position of the array. The slide containing the gel matrix was washed with sodium borohydride as in Example 3. This effected the reduction of Schiff bases and hence the covalent immobilization of a fraction of the oligonucleotide in the matrix by the loop of the hairpin structure. The immobilized self complementary oligonucleotide probe was partially duplex with a 3' overhang. It also had a 5' phosphate group. After immobilization, the array was hybridized with a fluorescent target DNA molecule that was complementary at its 3' end to the 3' overhang of the immobilized partially duplex probe. The array was incubated with a ligase enzyme that was expected to ligate the target molecule to the probe (since there was a perfect complementary match between the target and the single stranded overhang of the probe). After washing away unligated target molecules and drying of the matrix, the slide was scanned with a fluorescent scanner.

A portion of the resulting image is shown in Figure 14 with a profile of the fluorescent intensity to indicate the distribution of the fluorescent target in one dimension across three of the spots with immobilized partially duplex oligonucleotide probe. It can be appreciated from Figure 14 that the fluorescent target DNA molecule hybridized and was ligated into the spots containing the immobilized probe, and that the oligonucleotide was immobilized in discrete spots which are very uniform. The spots have a diameter of about 200 micrometers. Figure 15 shows a comparison of the relative amount of fluorescent DNA target that was ligated when the experiment was performed with a polymer matrix substrate and when it was performed in exactly the same way except that the amine-derivatized oligonucleotide probe was immobilized on glass surfaces activated either with diisothiocyanate or with aldehyde groups. In both cases the amount of target ligated to the probe was substantially greater with the polymer matrix than the activated planar glass surfaces.

Example 7

A fluorescently labeled protein, streptavidin, was dispensed onto the surface of an activated (aldehyde containing) gel as in Example 1, except that only a single droplet (350 picoliters) of the protein solution (100 microgram/milliliter) was dispensed onto each position of the array. The gel matrix was reduced with pyridine-borane as in Example 2. After washing away free protein and drying of the matrix, the slide was scanned with a fluorescent scanner. A portion of the resulting image is shown in Figure 16 with a profile of the fluorescent intensity to indicate the distribution of the fluorescent protein in one dimension across four of the spots. It can be appreciated from Figure 16 that the protein was immobilized in discrete spots. The spots have a diameter of about 180 micrometers.

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. An array comprising a plurality of addresses, each address of the plurality being positionally distinguishable from each other address of the plurality; and each of said plurality of addresses being located on, or in, a continuous porous matrix , wherein each of said plurality of addresses comprises a unique reagent.

2. The array of claim 1, wherein the unique reagent is covalently coupled to the continuous porous matrix .

3. The array of claim 1, wherein the plurality of addresses comprises at least 100 addresses.

4. The array of claim 1, wherein the plurality of addresses comprises less than 100 addresses.

5. The array of claim 1, wherein the array comprises at least 100 addresses per square centimeter of matrix surface.

6. The array of claim 1, wherein the unique reagent is a capture probe.

7. The array of claim 6, wherein the capture probe comprises a unique region.

8. The array of claim 1, wherein the array comprises a continuous surface.

9. The array of claim 1, wherein the plurality of addresses occupies a portion of a continuous porous array, which has a continuous surface.

10. The array of claim 9, wherein the plurality of addresses is at least 100 addresses.

11. The array of claim 1, wherein the addresses are imbibed into the matrix.

12. A method of making an array comprising a plurality of unique reagents disposed therein or thereon, comprising:

(1) providing a continuous porous matrix comprising a plurality of addresses, each address of the plurality being positionally distinguishable from each other address of the plurality; and each of said plurality of addresses being located on or in the matrix and;

(2) introducing a first unique reagent at a first address; and

(3) introducing a second unique reagent at a second address, thereby providing an array having a plurality of unique reagents disposed therein or thereon.

13. The method of claim 12, further comprising repeating step (3) until an array of unique reagents comprising diverse sequences are formed.

14. The method of claim 12, wherein the unique reagent is covalently coupled to the continuous porous matrix .

15. The method of claim 12, wherein the plurality of addresses comprises at least 100 addresses.

16. The method of claim 12, wherein the plurality of addresses comprises less than 100 addresses.

17. The method of claim 12, wherein the array comprises at least 100 addresses per square centimeter of matrix surface.

18. The method of claim 12, wherein the unique reagent is a capture probe.

19. The method of claim 12, wherein the capture probe comprises a unique region.

20. The method of claim 12, wherein the array comprises a continuous surface.

21. The method of claim 12, wherein the plurality of addresses occupies a portion of a continuous porous array, which has a continuous surface.

22. The method of claim 21, wherein the plurality of addresses is at least 100 addresses.

23. The method of claim 12, wherein the addresses are imbibed into the matrix.

24. A method of analyzing a sample comprising,

(1) providing an array comprising a plurality of addresses, each address of the plurality being positionally distinguishable from each other address of the plurality; and each of said plurality of addresses being located on a continuous porous matrix);

(2) contacting a molecule in a sample, with the array; and

(3) evaluating an interaction of the molecule of the sample with a reagent at at least one address of the plurality, thereby analyzing the sample.

25. The method of claim 24, wherein the sample is selected from a group consisting of a DNA molecule, all or part of a known gene; wild type DNA; mutant DNA; a genomic fragment, particularly a human genomic fragment; a cDNA, or a human cDNA.

26. The method of claim 24, wherein the address is evaluated by measuring a signal emitted from the address. The method of claim 24, wherein the unique reagent is covalently coupled to the continuous porous matrix .

27. The method of claim 24, wherein the plurality of addresses comprises at least 100 addresses.

28. The method of claim 24, wherein the plurality of addresses comprises less than 100 addresses.

29. The method of claim 24, wherein the array comprises at least 100 addresses per square centimeter of matrix surface.

30. The method of claim 24, wherein the unique reagent is a capture probe.

31. The method of claim 24, wherein the capture probe comprises a unique region.

32. The method of claim 24, wherein the array comprises a continuous surface.

34. The method of claim 24, wherein the plurality of addresses occupies a portion of a continuous porous array, which has a continuous surface.

35. The method of claim 24, wherein the plurality of addresses is at least 100 addresses.

36. The method of claim 24, wherein the addresses are imbibed into the matrix.

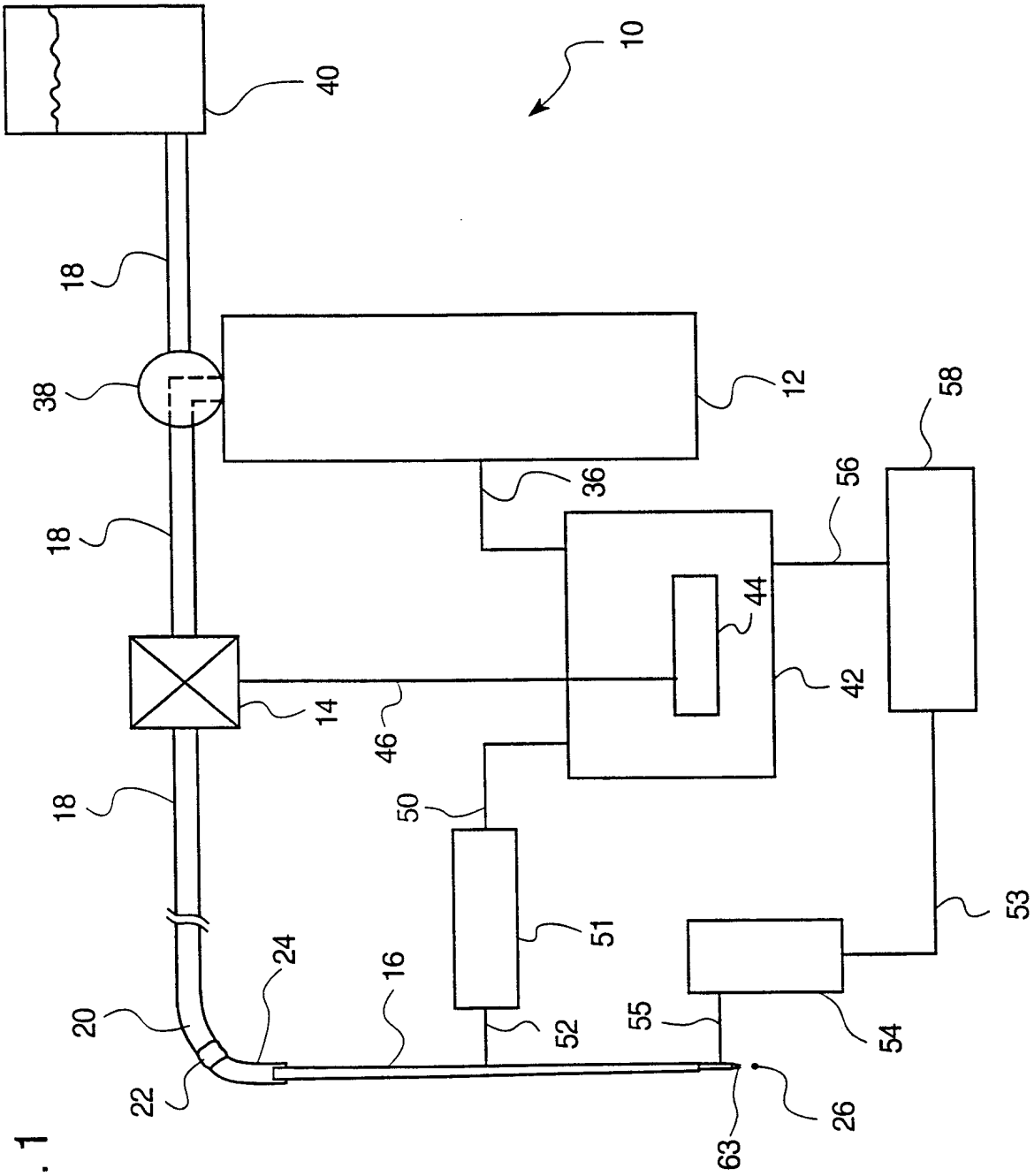
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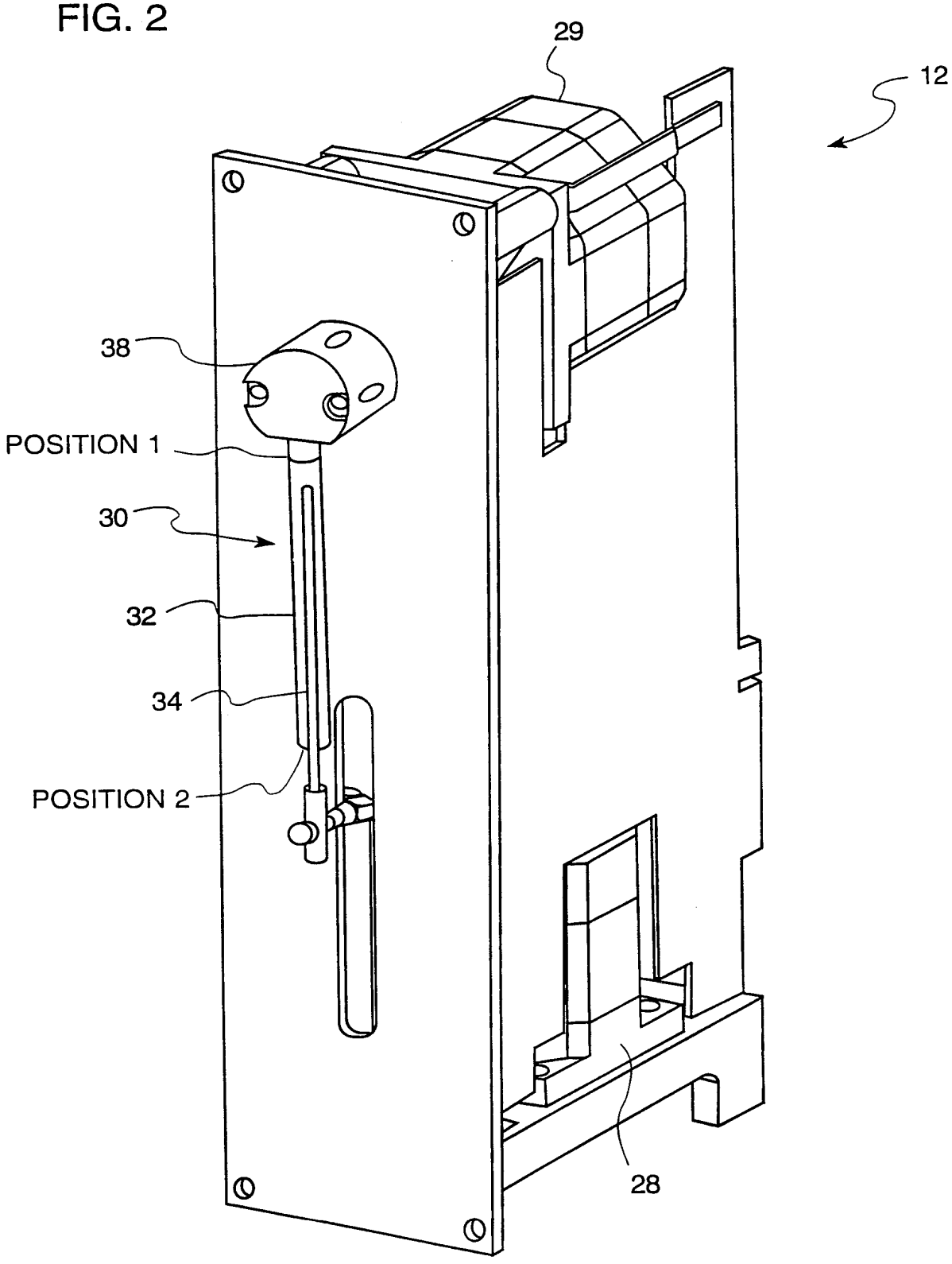
FIG. 1

FIG. 2



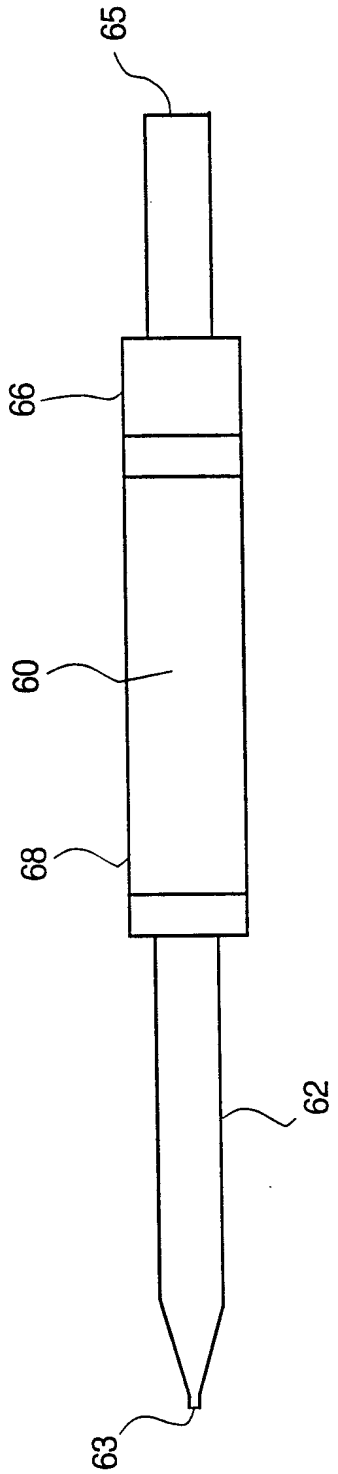


FIG. 3

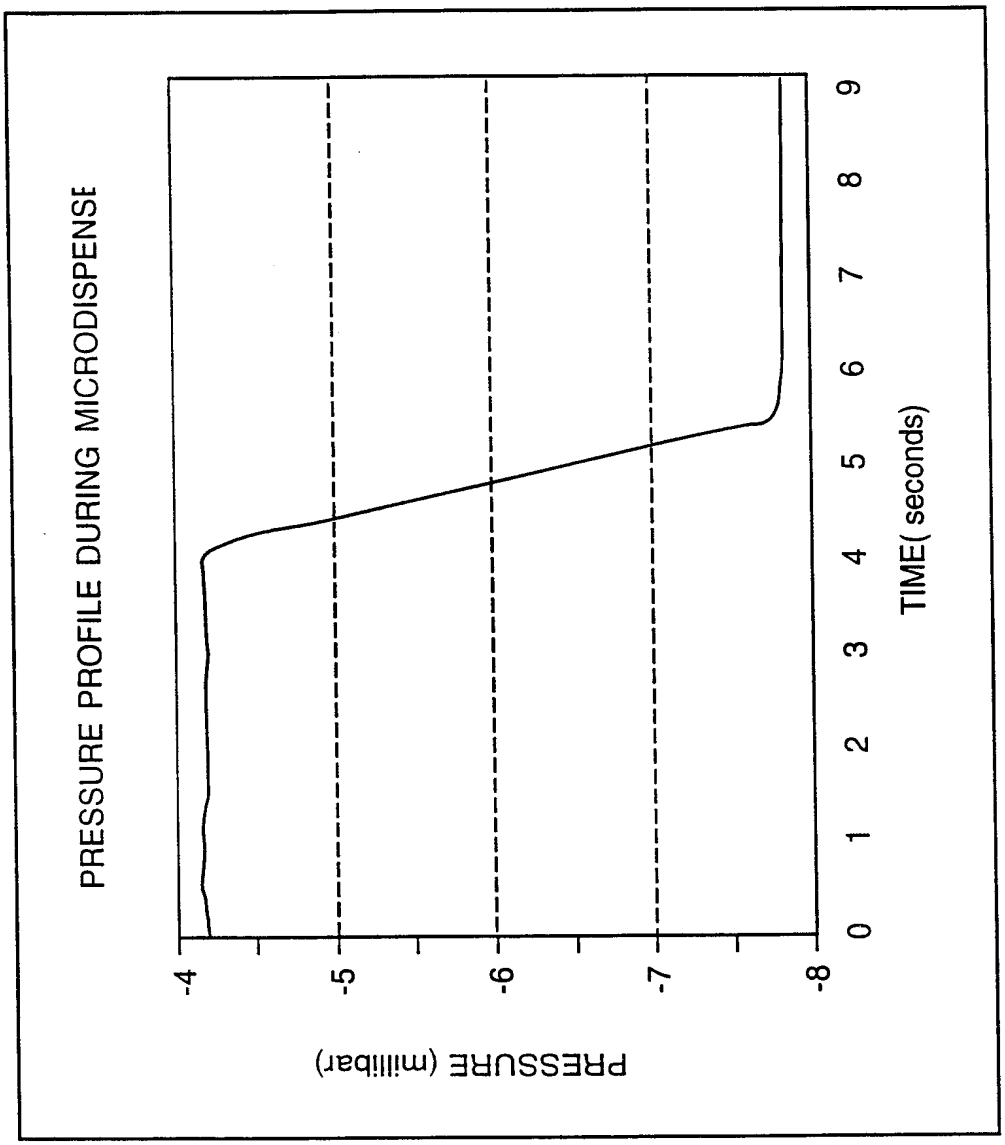
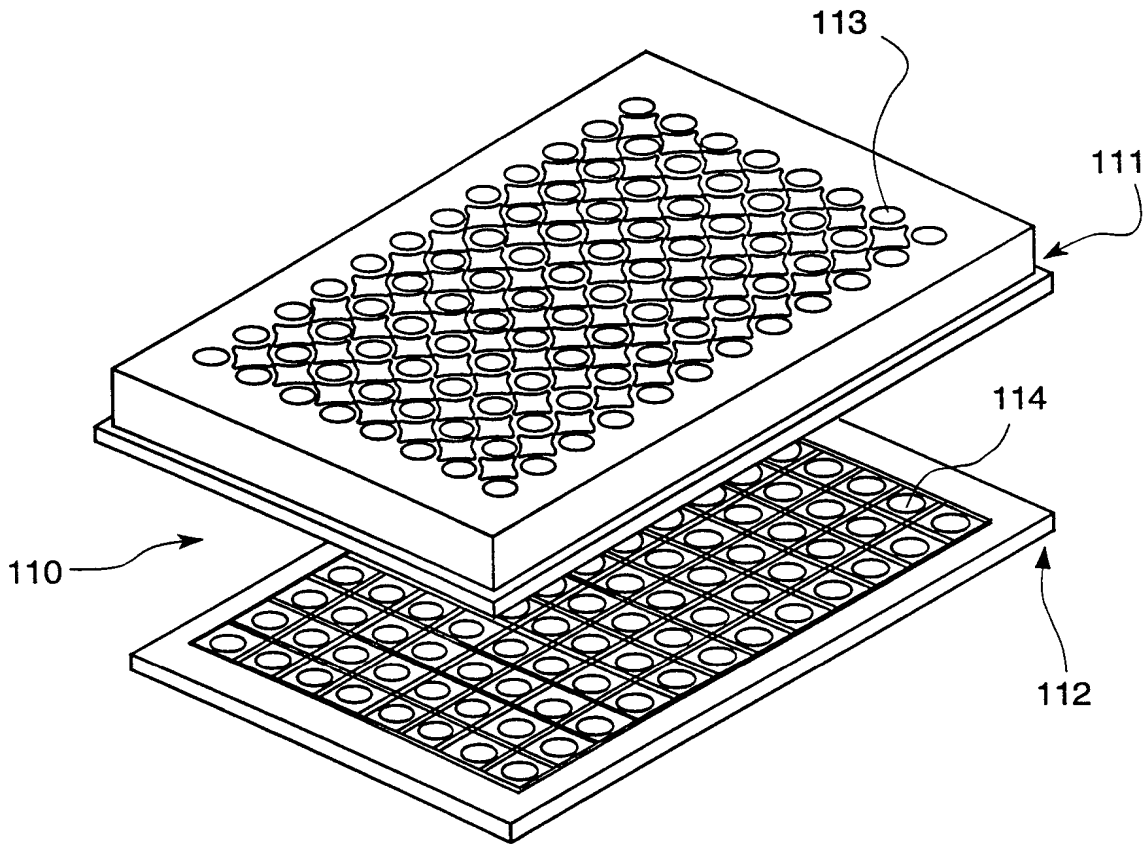

PRESSURE PROFILE DURING MICRODISPENSE
PRESSURE (millibar)
-4
-5
-6
-7
-8
0 1 2 3 4 5 6 7 8 9
TIME(seconds)

FIG. 4

FIG. 5



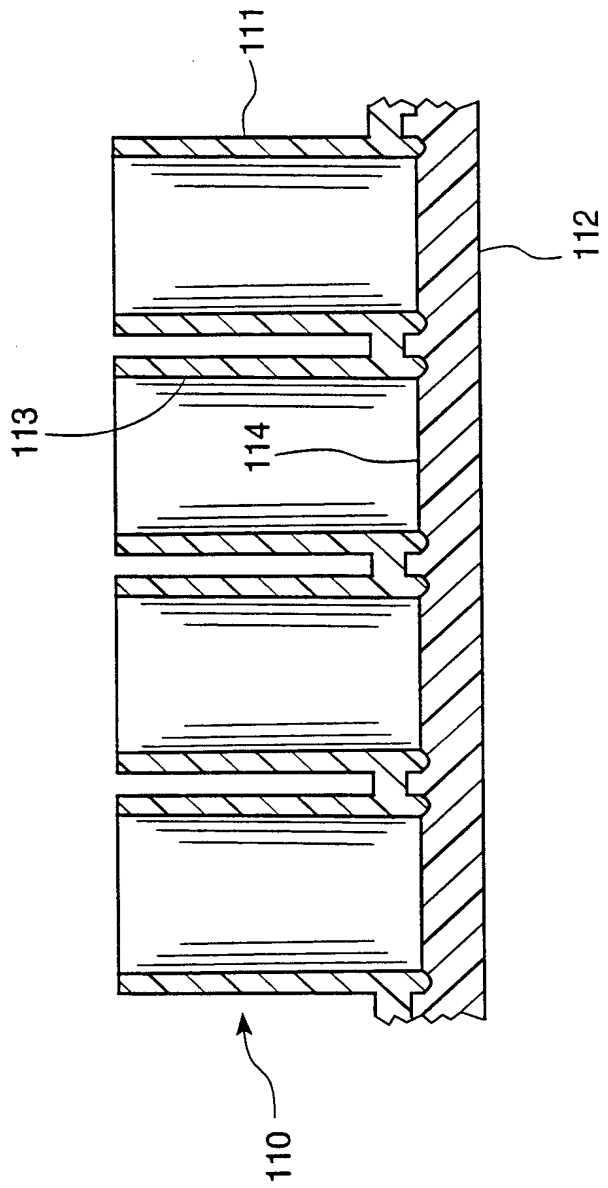


FIG. 6

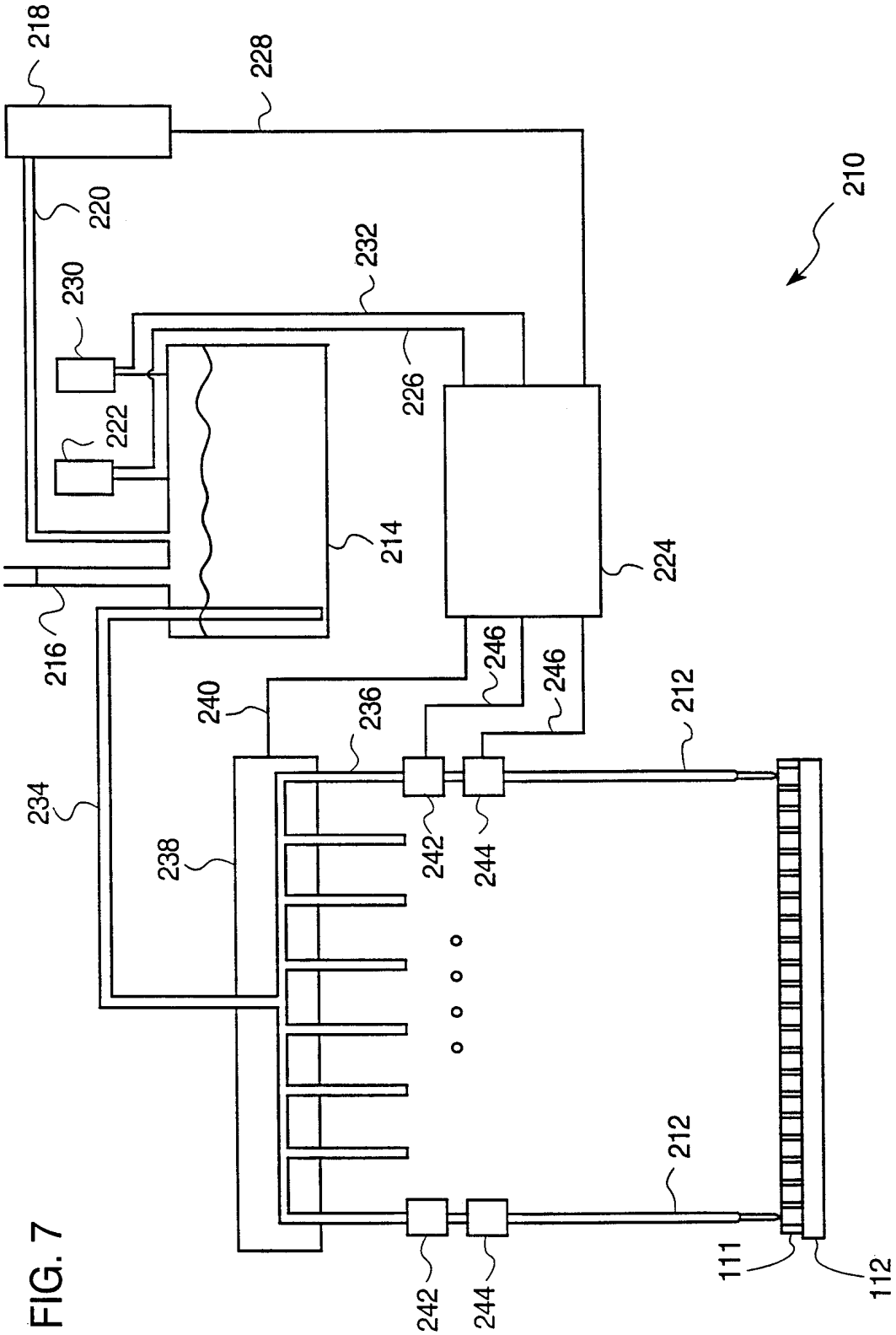

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FIG. 7

FIG. 8

FIG. 9

FIG.10

FIG. 11a

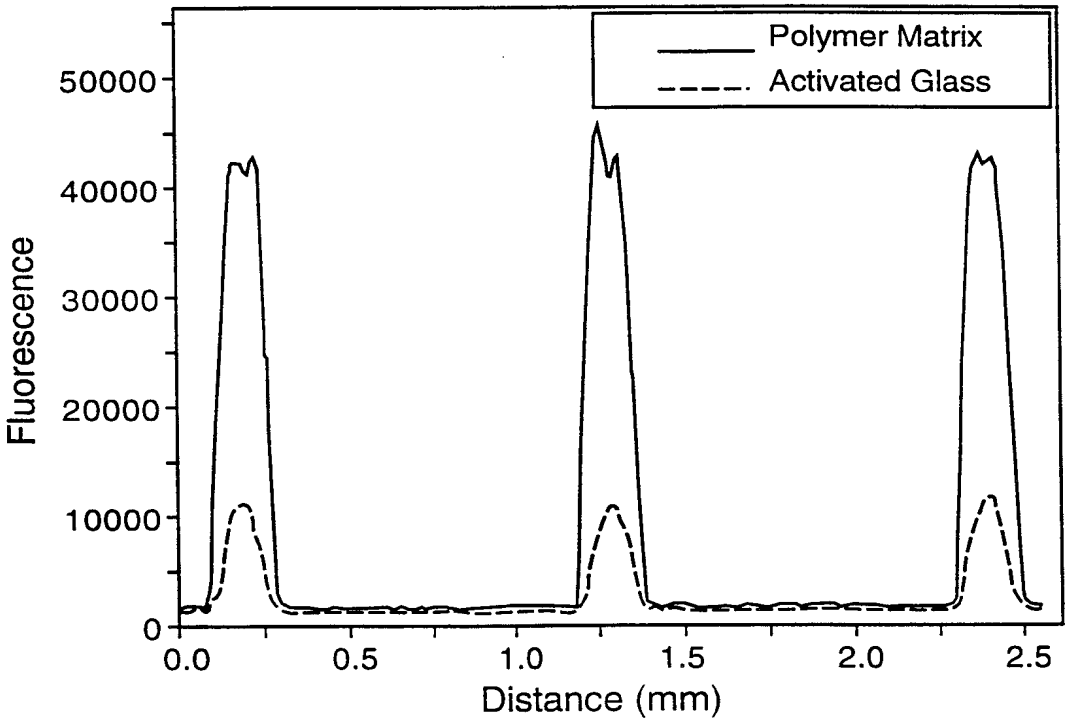

FIG. 11B

FIG. 12A

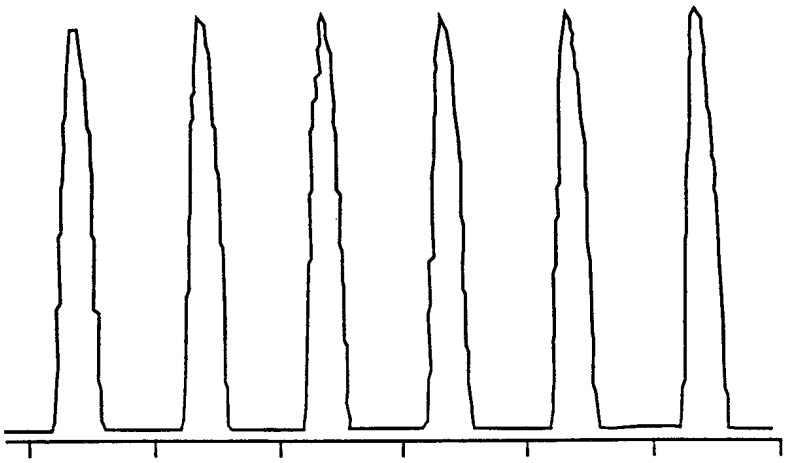

FIG. 12B

FIG. 13

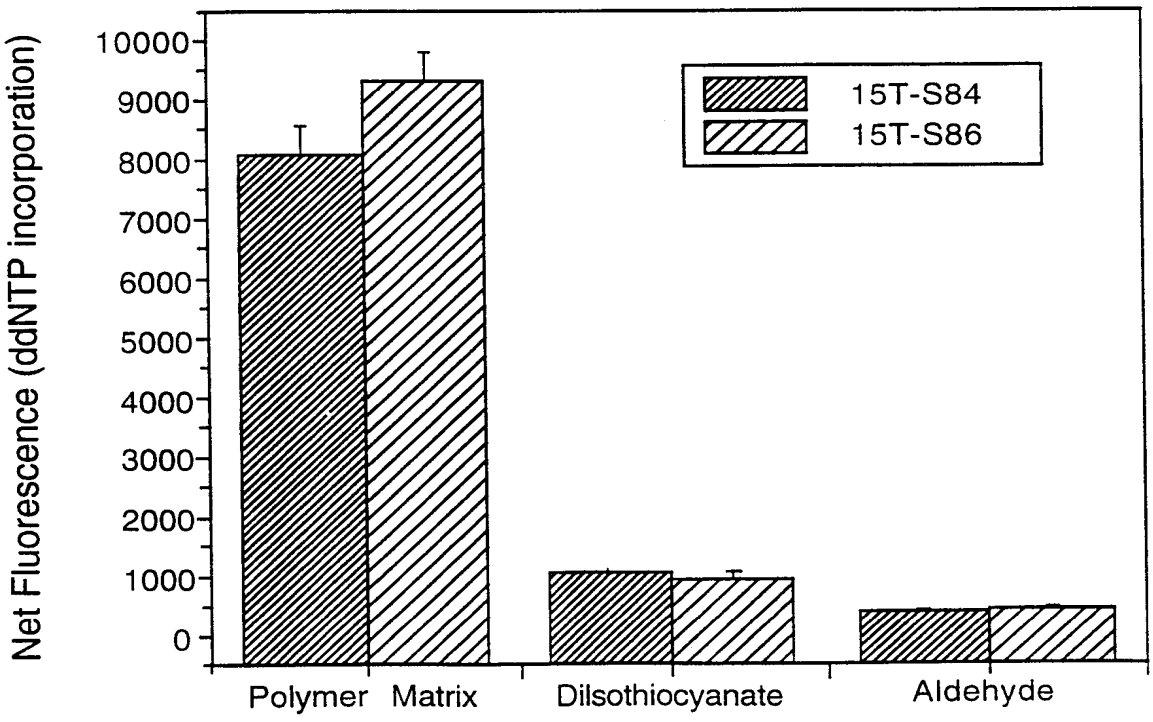

FIG. 14A

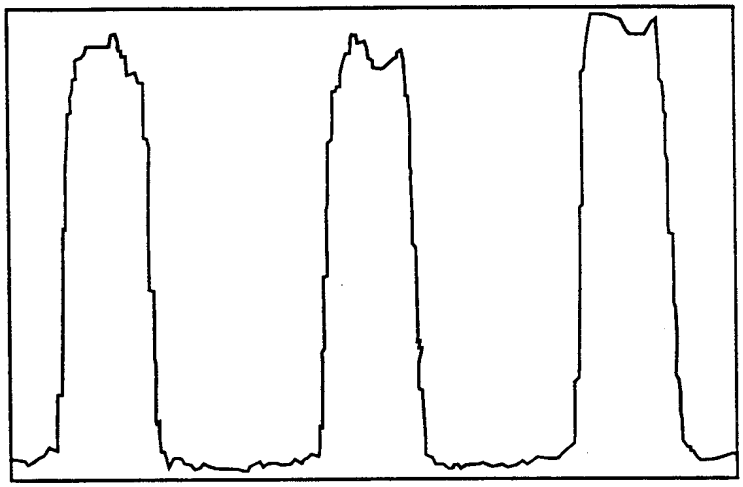

FIG. 14B

FIG. 15

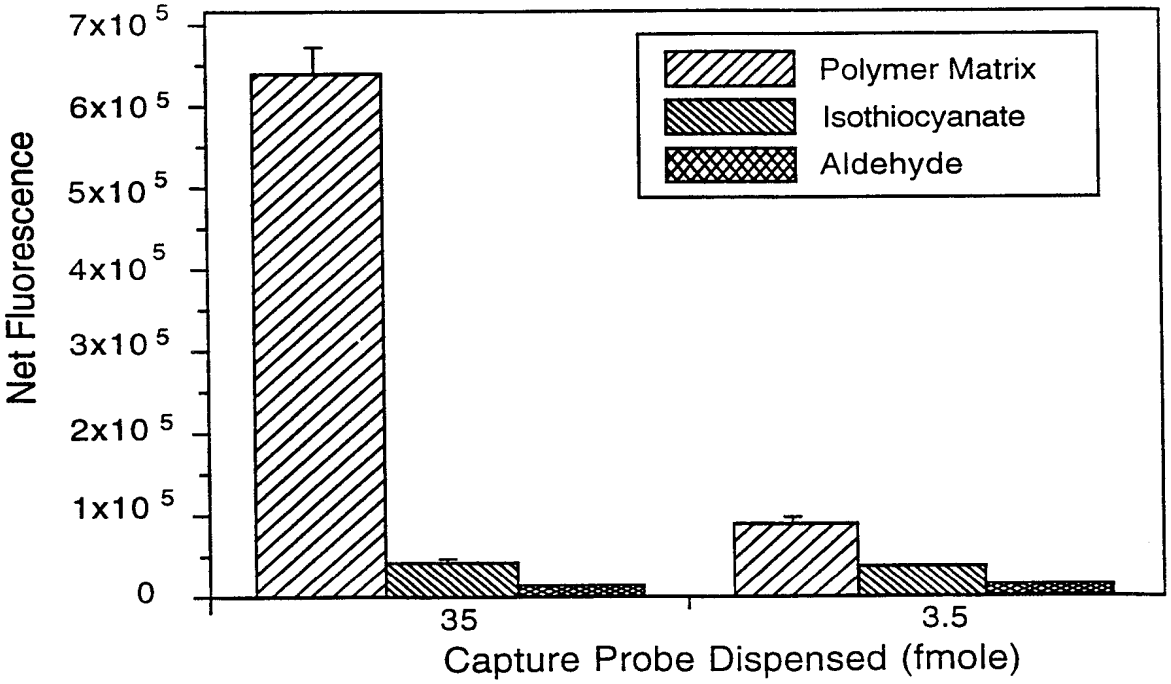

FIG. 16A

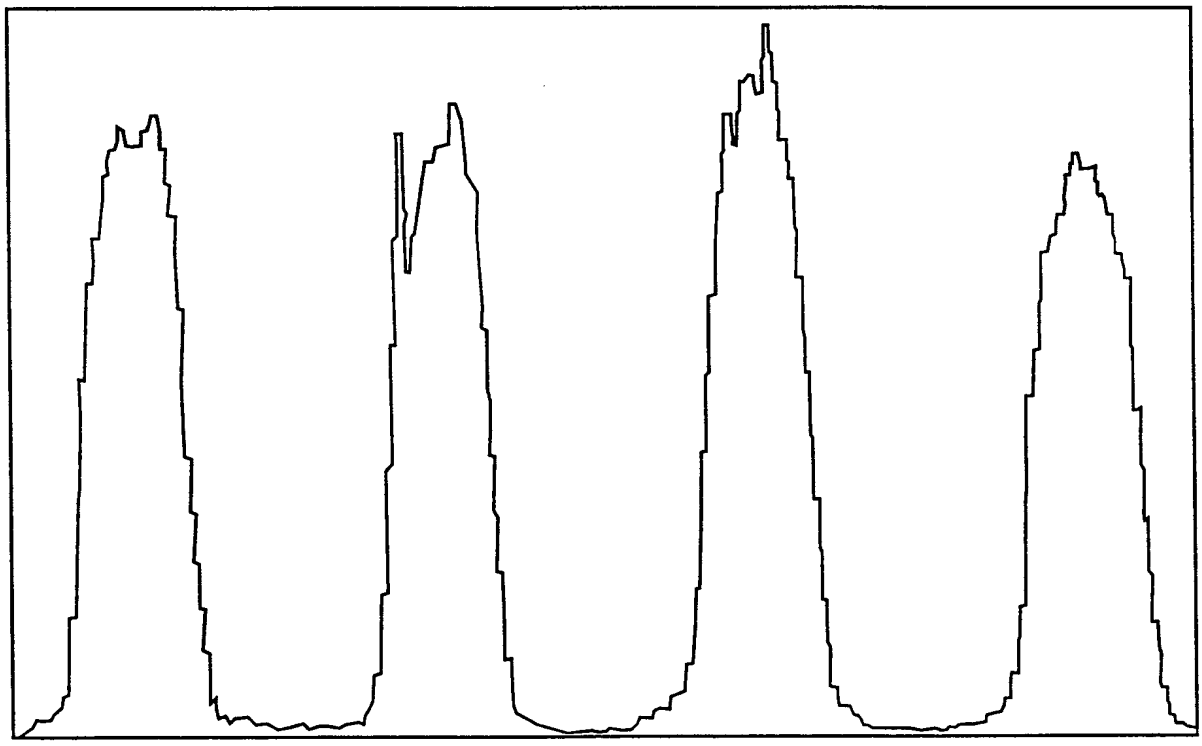

FIG. 16B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/07796

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68
US CL :435/6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/50,68.1;435/6; 536/25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 4,656,127 A (MUNDY) 07 April 1987, see especially Figures 4 and 8 and related description.	1, 4, 6-9, 12, 13, 16, 18-21, 24, 25, 28, 30-34 --- 1-10, 12-22, and 24-35

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 11 JULY 2000	Date of mailing of the international search report 04 AUG 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ARDIN MARSCHEL Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/07796

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	FRANK, R. Strategies and Techniques in Simultaneous Solid Phase Synthesis Based on the Segmentation of Membrane Type Supports. Bioorganic & Medicinal Chemistry Letters. 1993, Volume 3, Number 3, pages 425-430, see especially Figures 3A and 5A and related description.	1, 4, 6-9, 12, 13, 16, 18-21, 24, 25, 28, and 30-34 --- 1-36
Y	GB 2,156,074 A (ORION-YHTYMA OY [FINLAND]) 02 October 1985, see entire document.	1-10, 12-22, and 24-35
X --- Y	WO 95/25116 A1 (CALIFORNIA INSTITUTE OF TECHNOLOGY) 21 September 1995, see especially page 6, lines 8-26.	1-3, 5-10, 12-15, 17-22, 24, 25, 27, and 29-35 --- 1-10, 12-22, and 24-35
Y	US 5,871,928 A (FODOR et al.) 16 February 1999, see entire document.	1-10, 12-22, and 24-35
Y	US 5,700,637 A (SOUTHERN) 23 December 1997, see entire document.	1-10, 12-22, and 24-35
X --- Y	US 5,847,105 A (BALDESCHWIELER et al.) 08 December 1998, see especially Figure 1B.	1-3, 5-10, 12-15, 17-22, 24, 25, 27, and 29-35 --- 1-10, 12-22, and 24-35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/07796

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,744,305 A (FODOR et al.) 28 April 1998, see entire document.	1-10, 12-22, and 24-35
X --- Y	US 5,807,522 A (BROWN et al.) 15 September 1998, see especially column 12, lines 1-15.	1-3, 5-10, 12-15, 17-22, 24, 25, 27, and 29-35 --- 1-10, 12-22, and 24-35
X --- Y	WO 95/35505 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 28 December 1995, see especially the bridging paragraph between pages 23 and 24.	1-3, 5-10, 12-15, 17-22, 24, 25, 27, and 29-35 --- 1-10, 12-22, and 24-35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/07796

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST and Dialog covering EMBASE, MEDLINE, BIOTECH ABSTRACTS, WPI, over terms: array, porous, unique, hybridize, assay, covalent, and imbibed